EXPRESSION IN PLANTS OF ANTIBODIES AGAINST ENTEROTOXIGENIC *ESCHERICHIA COLI*

Background

Enterotoxigenic *Escherichia coli* (enterotoxigenic *E. coli*; ETEC) are a major cause of acute diarrheal disease in both humans and animals worldwide. Infectious diarrhea of neonatal animals is one of the most common and economically devastating conditions encountered in the animal agriculture industry (Holland, *Clin Microbiol. Rev.*, 3:345, 1990). Two virulence attributes that characterize ETEC are colonization of the small intestine surface and the production of enterotoxins. ETEC produce two types of enterotoxins heat labile enterotoxins (LT) and heat stable enterotoxins (ST). The LT is closely related to cholera toxin and is an oligometric protein of 85 kDa composed of five identical B subunits arranged in a ring and one A subunit. In contrast, the STs are small, monomeric toxins with a molecular weight of approximately 5 kDa. STs are divided into two different classes, ST-I and ST-II (Fassano, *Gut*, 50(Suppl III):III9-III14, 2002).

The other essential virulence attributes are the structural components of the bacterial cells that allow them to colonize the intestinal mucosa. The most important of these are the pili or fimbriae. The pathogenesis involves binding of the fimbriae to glycoprotein or glycolipid receptors on mucus and apical cell surfaces of villous epithelial cells, and the induction of diarrhea, largely through the effects of enterotoxins. Fimbriae are identified antigenically by number. The main antigens expressed by ETEC of livestock include K88 (F4), K99 (F5), 987P (F6), F18ac (previously called F107ac and 2134P), and F41. In humans, the antigens are referred to a colonization factors (CFs). Most CFs are fimbrial proteins and consist of a single CFA/I, while others such as CFA/II and CFA/IV are formed by a complex of different antigens, termed coli surface antigens (CS) (Wiseman et al., *Emerging Infectious Diseases*, 5:395-403, 1999).

The increased use of intensive management and artificial rearing practices in livestock have increased the incidence of clinical ETEC infections in young livestock. The loses due to enterotoxin-induced diarrheal disease can be significant especially among animals infected during the first four days after birth. As animals age, they naturally acquire the ability to withstand infections by ETEC, although in swine ETEC

infections can cause problems up to 5 weeks of age. During the neonatal period, however, livestock have no natural defenses against ETEC and mortality rates can reach 70% in affected animals. Suckling neonates can be passively immunized by ingestion of maternal antibodies in colostrum, but because adult animals are insensitive to ETEC fimbrial proteins, antibody titers in adult cows, ewes and sows tend to be low or non-existent. In an attempt to booster transfer of maternal antibodies, prepartum vaccination of dams in late gestation against ETEC fimbrial antigens has been used (Acres et al., *Infect. Immun.* 25:121-126, 1979; Nagy *Infect. Immun.*, 27:21-24, 1980). Although successful, this method is limited by the need for multiple prepartum vaccinations, the need to accurately determine when in gestation vaccination should occur, and the inherent variability in immune response between dams to vaccination. In addition, this method is not suitable for use with human subjects or for adults, such as the prevention of traveler's diarrhea in humans.

An alternative method is passive immunization by the administration of a composition containing antibodies against ETEC. Because ETEC reside in the small intestine, oral administration of antibodies has been used. A review of oral administration of antibodies to treat enteric pathogens can be found in Reilly et al., *Clin. Pharmacokinet.*, 32:313-323, 1997 and Carlander et al., *Immunol. Res.*, 21:1-6, 2000.

Results with passive immunization against ETEC have been mixed. U.S. Patent No. 4,652,448 discloses a method for the prevention and treatment of enterotoxin-induced diarrhea in animals by the administration of a monoclonal antibody obtained from hybridoma cells (U.S. Patent 4,443,549). Tacket et al., *N. Engl. J. Med.*, 318:1240-1243, 1988, reported that oral administration of lyophilized immunoglobulins prepared from colostrum or cows immunized with several ETEC serotypes provided protection against challenge with ETEC H10407 (078:H11). Sherman et al., *Infect. Immun.*, 42:653-658, 1983, found that oral administration of mouse ascitic fluid containing a mouse K99 monoclonal antibody reduced the severity and mortality, but not the incidence of diarrhea in calves. Yokoyama et al., *Am. J. Vet. Res.*, 54:867-872, 1992, reported that oral administration of egg yolk IgG provided protection against K88 challenge when administered daily at high dosages. The egg IgG survived well in newborn and pre-weaning pigs, but disappeared quickly in 28-day-old pigs. In a similar study, Yokoyama et al., found that thrice daily oral administration of dried egg yolk powder from hens immunized against ETEC over a three day period, decreased

mortality to an ETEC challenge in a dose dependent manner. Freedman et al., *J. Infect. Dis.*, 177:662-667, 1998, reported that oral administration in conjunction with sodium bicarbonate of lyophilized antibody concentrate from the milk of cows immunized against *E. coli* provided protection against clinical diarrhea following oral challenge with ETEC. Additional examples of oral administration of antibodies can be found in U.S. Patent Nos. 3,984,539; 4,096,244; 4,623,541; and 4,816,252.

In contrast, Casswall et al., *Scand. J. Gastroenterol.* 7:711-718, 2000, found that oral administration of bovine milk immunoglobulin concentrate from cows hyperimmune cows had no significant therapeutic benefit in the treatment of acute diarrhea due to enterotoxigenic or enteropathogenic *E. coli*. Likewise, Tacket et al., *J Infect. Dis.*, 180:2056-2059, 2000, reported that oral administration of a partially enteric-coated preparation of concentrated immunoglobulins from cow's milk failed to protect against a challenge with ETEC. These contradictory results may be due in part to the significant variability in the affinity of different monoclonal antibodies for a given target. This variability can produce anomalous results unless definitive and optimal biding characteristics are biochemically assayed.

Although reportedly successful in many instances, it has been recognized in the art that commercial development for products for passive immunization against ETEC have been limited by the lack of methods for producing large quantities of specific antibodies (Freedman et al., *J. Infect. Dis.*, 177:662-667, 1998).

Plants provide an efficient and economical means to produce large amounts of protein. Reviews of the use of plants to produce medically useful proteins can be found, for example, in Walmsley and Arntzen, *Curr. Opin. Biotechnol.*, 11:126-129, 2000; Doran, *Curr. Opin. Biotechnol.*, 11:199-204, 2000; Giddings et al., *Nature Biotechnol.*, 18:1151-1155, 2000; and Djaniell et al., *Trends Plant Sci.*, 6:219-226, 2001. There have been numerous reports of the successful production of mammalian antibodies in plants. Some examples of these include, Zeitlin et al., *Nature Biotech.*, 16:1361-1364, 1998; Artsaenko et al., *Molec. Breeding*, 4:313-319, 1998; Stoger et al., *Plant Molec. Biol.*, 42:583-590, 2000; Chargelegue et al., *Transgenic Res.*, 9:187-194, 2000; Bakker et al., *Proc. Natl. Acad. Sci. USA*, 98:2899-2904, 2001; U.S. Patent Nos. 5,202,422; 5,639,947; 5,759,808; 5,840,526; 5,959,177; 5,990,385; 6,046,037; 6,020,169; 6,080,560; European Patents and Patent Applications EP0497904; EP1048734; EP1118669; and PCT Publications WO9106320; WO9742313; and WO0025574.

What is needed, therefore, is not only an economical way of producing antibodies directed against enteric pathogens in large quantities, particularly when such antibodies are effective in preventing and/or treating such pathogens when enterally administered in an unpurified, partially purified or substantially purified form. The present invention addresses this need by producing such antibodies, or fragments thereof, in plants.

Summary

The present invention provides *inter alia* polynucleotides encoding immunoglobulin light chains and/or heavy chains and fragments thereof, which recognize enteric pathogens. In particular such light and heavy chains and fragments thereof are specific for the K88 or K99 antigens of *E. coli.*

Among the several aspects of the invention is provided an isolated polynucleotide comprising SEQ ID NO. 1 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 1 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

Another aspect provides an isolated polynucleotide comprising SEQ ID NO. 3 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 3 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

A further aspect provides an isolated polynucleotide comprising SEQ ID NO. 2 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 2 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

Yet another aspect provides an isolated polynucleotide comprising SEQ ID NO. 4 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 4 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

Another aspect provides an isolated polynucleotide comprising SEQ ID NO. 5 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 5 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

Still another aspect provides an isolated polynucleotide comprising SEQ ID NO. 7 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or

at least 99% sequence identity or homology with SEQ ID NO. 7 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

Yet another aspect provides an isolated polynucleotide comprising SEQ ID NO. 9 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 9 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

Another aspect provides an isolated polynucleotide comprising SEQ ID NO. 11 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 11 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

A further aspect provides an isolated polynucleotide comprising SEQ ID NO. 13 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 13 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the

preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

Another aspect provides an isolated polynucleotide comprising SEQ ID NO. 15 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 15 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a heavy chain of an immunoglobulin.

An additional aspect provides an isolated polynucleotide comprising SEQ ID NO. 6 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 6 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

Another aspect provides an isolated polynucleotide comprising SEQ ID NO. 8 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 8 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one

aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

Still another aspect provides an isolated polynucleotide comprising SEQ ID NO. 10 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 10 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

A further aspect provides an isolated polynucleotide comprising SEQ ID NO. 12 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 12 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

Yet another aspect provides an isolated polynucleotide comprising SEQ ID NO. 14 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 14 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

Another aspect provides an isolated polynucleotide comprising SEQ ID NO. 16 or the complement thereof. One embodiment provides an isolated polynucleotide that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity or homology with SEQ ID NO. 16 or its complement. Another embodiment provides an isolated polynucleotide comprising at least 15 nucleotides that hybridizes under stringent or highly stringent conditions with any of the preceding isolated polynucleotides. In still another embodiment, the preceding isolated polynucleotides encode at least one variable region of an immunoglobulin. In one aspect, the polynucleotides encode at least one variable region of a light chain of an immunoglobulin.

A further aspect provides an isolated polypeptide encoded by any of the isolated polynucleotides of the invention. Also included are polypeptides that have at least at least 50%, at least 60%, 70%, 80%, 90%, 95% or 99% amino acid sequence identity with the polypeptides encoded by any of the polynucleotides of the present invention and which are capable of binding to an antigenic epitope. Still another embodiment includes polynucleotides which have undergone conservative amino acid substitutions. In an additional embodiment, the polynucleotides of the present invention have been codon optimized for expression in a plant. In one embodiment, the codons have been optimized for expression in a monocotyledonous plant. In another embodiment, the codons have been optimized for expression in corn. In a further embodiment the codons have been optimized for expression in a dicotyledenous plant. In still another embodiment, the codons have been optimized for expression in a plant plastid, for example, a chloroplast.

An additional aspect provides recombinant vectors comprising at least one of any of the polynucleotides of the invention. In one embodiment, the vectors are cloning vectors, while in another embodiment the vectors are expression vectors. One aspect provides recombinant expression cassettes comprising as operably linked components, at least one promoter, at least one of any of the polynucleotides of the present invention, and at least one termination sequence. In an additional embodiment, the expression cassette further comprises and endoplasmic reticulum (ER) retention signal operably linked to any of the polynucleotides of the present invention.

Still further aspects provide host cells comprising at least one of the recombinant vectors or expression cassettes of the present invention, as well as host cells expressing

an immunoglobulin molecule, or fragment thereof, as described herein. The host cells can be bacterial cells, fungal cells, yeast cells, algae, plant cells, or animal cells. In one embodiment, the host cells are plant cells. In another embodiment, the host cells are monocot plant cells. In a further embodiment the host cells are dicot plant cells. In still another embodiment, the host cells are cereal plant cells. In yet another embodiment, the recombinant vector or expression cassette is contained in, and/or an immunoglobulin molecule of the present invention is expressed in, the plastid or organelle of the host cell. An additional embodiment encompasses plants comprising a host cell of the present invention. Methods of producing such host cells and plants form yet further aspects of the invention.

A further aspect provides a recombinant nucleotide construct encoding an immunoglobulin containing a light chain and a heavy chain, wherein said heavy chain is encoded by SEQ ID NO. 1 or 3 and the light chain is encoded by SEQ ID NO. 2 or 4. In one embodiment, the immunoglobulin is directed against the K99 antigen of an enterotoxigenic *E. coli* bacteria. In another embodiment, the construct further comprises an endoplasmic reticulum (ER) retention signal.

An additional aspect provides a recombinant nucleotide construct encoding an immunoglobulin containing a light chain and a heavy chain, wherein the heavy chain is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs. 5, 7, 9, 11, 13 and 15, and the light chain is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs. 6, 8, 10, 12, 14 and 16. In one embodiment, the immunoglobulin is directed against the K88 antigen of an enterotoxigenic *E. coli* bacteria. In another embodiment, the construct further comprises an endoplasmic reticulum (ER) retention signal.

The ability to increase yield and stability (e.g. molecular half-life) of fully assembled and functional antibody products is important. One way in which at least the stability of such products can be achieved, is through creating a stabilized C_H3 or Fc domain. Such a stabilised domain may then be useful as a platform for functional design, in particular where such functional design involves mutagenising the antibody product. Starting from an ultra-stable platform provides more room for compromise when mutating an antibody molecule for functional purposes.

Thus, a further aspect of the invention provides an immunoglobulin molecule, particularly the heavy chain, comprising a C_H3 domain that contains at least one

mutation selected from the group consisting of S174G, Y179D, G197K, G197A, S207G and T246L. In one embodiment, the immunoglobulin is an isolated immunoglobulin. In another embodiment, the heavy chain is a bovine heavy chain. Also included are isolated polynucleotides encoding such mutated C_H3 domains. The mutated C_H3 domain can be combined with any of the heavy or light chain variable regions disclosed herein.

Another aspect provides an immunoglobulin molecule, particularly the heavy chain, comprising a C_H2 domain that contains at least one mutation selected from the group consisting of N85H, R109P, T116L, and H126N. In one embodiment, the immunoglobulin is an isolated immunoglobulin. In another embodiment, the heavy chain is a bovine heavy chain. Also included are isolated polynucleotides encoding such mutated C_H2 domains. The mutated C_H2 domain can be combined with any of the heavy or light chain variable regions disclosed herein. These mutation enhance overall stability of the domain and provide an ultrastable platform for functional design and can be grafted into full-length antibody sequences to increase molecular yield and half-life.

A further aspect provides an immunoglobulin molecule comprising a mutated C_H2 and/or C_H3 domain disclosed herein. In one embodiment, the immunoglobulin molecule is an isolated immunoglobulin molelcue. In another embodiment, the immunoglobulin molecule containing the mutated C_H3 and/or C_H2 domain is combined with of the variable regions disclosed herein.

Another aspect provides a transgenic plant comprising at least one of the polynucleotides disclosed herein. In one embodiment, the plant expresses an antibody or an antibody fragment encoded by one of the polynucleotides disclosed herein. In another embodiment, the antibody or antibody fragment expressed is a single chain antibody, a scFv, or a VHH antibody. In a further embodiment, the antibody or antibody fragment binds to the K88 or K99 antigen of ETEC. Method of producing such plants are also considered as further aspects of the invention.

Yet another aspect provides a method of treating or preventing an enteric disease in an animal comprising enterally administering an effective amount of a compound containing material or partially purified material from any of the transgenic plants comprising any of the polynucleotides disclosed herein. In one embodiment, the material or partially purified material comprises leaf material, seed material, fruit material, root material, stem material or any combination thereof. In another

embodiment, the antibodies or antibody fragments are purified from the plant material prior to enteral administration. In still another embodiment, enteral administration comprises oral administration. In an additional embodiment, the plant material, partially purified plant material or purified antibody from the plant material is administered in a liquid. In one aspect, the plant material or partially purified plant material contains at least 0.1 mg of antibody per kg of plant material or partially purified plant material. In yet another embodiment, the animal is administered plant material, partially purified plant material or purified antibody form plant material disclosed herein containing at least 10 mg of antibody or antibody fragments per day.

A further aspect provides a composition comprising plant material or partially purified plant material or purified antibody or antibody fragments from any of the transgenic plants disclosed herein. The composition may be a pharmaceutical composition and may further comprise a pharmaceutically acceptable diluent or excipient. In one embodiment, the composition is an animal feed or human food product. In another embodiment, the composition is a milk replacer or infant formula in either liquid or powder form. In still another embodiment, the composition is a feed or food additive. In yet another embodiment, the composition is a nutritional supplement.

Brief Description of the Figures

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims and accompanying figures where:

Figure 1 shows the primers for the K99 V_H and V_L genes.

Figure 2 shows the construction of the anti-K88 kappa light and IgG1 heavy chain for maize optimized codon usage and synthesis: Agro-RB-*Hind*III-γZein promoter-BanHI-gZSS-SpeI-anti-K99 V1-Xho-constant light region-XmnI-SEKDEL-AgeI-PEPC intron 35S term-MluI-MluI-γZein Promoter-BamHi-gZSS-SpeI-anti-K99Vh CH1 aa 1-10-AccI-Chaa1-Hinge-CH2-Ch3-XmaI-SEKDEL-SacI-PEPC intron 35S term-KpnI-KpnI-Ubi promoter-BamHI=PMI plnat selection gene-SacI-NOS-LB Agro.

Figure 3 shows antibody expression in rice callus transformed with anti-K99 light and heavy chains (pTMR103 and pTMR105). The antibody expression was

detected by Western blot using reducing gells. NT: Non-transgenic tissue. Lanes 1-8: protein extract from 8 different events.

Figure 4 shows Western blot analysis of corn leaf samples from independent transgenic corn lines. Ten µg of total soluble proteins was loaded per lane. Lane 1: extract from corn leaf transformed with pTMR554 (with ER signal). Lanes 2, 3, 4: extract from corn leaf transformed with pTMR555 (without no ER). Lane NT: extract from non-transgenic corn leaf. Lane MW: Molecular Weight Marker (kD). Lane mab: 40 ng of anti-K88 antibody purified from hybridoma cells. Gel A, B, D: 3-8% (w/v) Tris-acetate NuPAGE non-reducing conditions; Gel A: Detection with rabbit anti-mouse H+L (1:500) followed by the alkaline-phosphatase goat anti rabbit H+L (1:4000). Gel B: Detection with alkaline-phosphatase anti-mouse Fc (1:2000). Gel D: Detection with rat anti-mouse kappa (1:200) followed by alkaline-phosphatase goat anti-rat H+L (1:5000). Gel C: Bis-Tris 4-12% reducing SDS-PAGE; Detection with the rabbit anti-mouse H+L (1:500) and with alkaline-phosphatase anti-mouse Fc (1:2000).

Figure 5a shows surface plasmon resonance evaluation of binding complex between plant-produced antibody and the K88 pili. Antibody purified from hybridoma or plant cells were analyzed by surface plamon resonance using a BIACORE 3000. The sensograms correspond to the injection of K88 antibody purified from corn leaf expressing pTMR554 (plmAb), K88 antibody purified from hybridoma cells (hybridoma 36/44). The blank run is shown in grey.

Figure 5b shows the Biacore analysis demonstrating the differential post-translational processing of recombinant IgG1 molecules based on organelle targeting. Both panels show injections of recombinant IgG1 onto an antigen coated chip. The maximum Response (y-axis) depends on the rate of binding and on the overall size of the molecule. **A.** Injection of IgG1 with and without the ER retention signal. +ER IgG1 is more concentrated, which is why it binds faster. However, the –ER IgG1 must have a slightly greater molecular weight (MW), possibly due to more extensive glycosylation, which explains how it generates a larger Response, even though the kinetics of its binding are slightly slower (due to its lower concentration). **B.** Injection of 9 individual, -ER, transgenic corn seed extracts. There is variation in the concentrations of IgG1 in each sample, the kinetic traces *NEVER CROSS*, because the MWs of each –ER IgG1 molecule are all exactly the same. Thus, post-translational modifications in the

plant samples depend highly on cellular compartmentalization and proteins targeted to the same cellular location appear to have identical posttranslational modifications.

Figure 6a shows concentration profiles of 7/46, 36/41 and 17/44 binding to the K88 chip. 6A. Displayed are the sensogram overlays of 0.5, 2, 5, 10, 20, 50, 100 and 250 nM 17/44 injections onto the K88-immobilized sensor chip. 6B. Overlays of 0.1, 0.2, 0.5, 1.0, 2, 5 and 10 nM 36/41 injections onto the K88 chip. 6C. Overlays of 5, 10 , 20, 50 and 100 nM 17/44 injections onto the K88 chip. All three profiles were fit to a bivalent analyte model. Residuals for each fit are displayed below each profile.

Figure 6b shows the concentration profile of anti-K99 monoclonal Antibody (purified from hybridoma cell cultures) was passed over a K99-coated surface plasmon resonance chip. The concentration profile was fit to a bivalent association in order to determine approximate association/dissociation rate constants as well as an overall equilibrium dissociation constant, KD.

Figure 7 shows the level anti-K99 antibodies in ileal digesta on Day 0 for the single dose treatment group.

Figure 8 shows the level anti-K99 antibodies in ileal digesta on Day 0 for the triple dose treatment group.

Figure 9 shows the level anti-K99 antibodies in ileal digesta on Day 1 for the single dose treatment group.

Figure 10 shows the level anti-K99 antibodies in ileal digesta on Day 1 for the triple dose treatment group.

Figure 11 shows the level anti-K99 antibodies in ileal digesta on Day 2 for the single dose treatment group.

Figure 12 shows the level anti-K99 antibodies in ileal digesta on Day 2 for the triple dose treatment group.

Figure 13 shows the three-day average levels of anti-K99 antibodies in ileal digesta.

Figure 14 shows the level of IgG in ileal digesta on Day 0 for the single dose treatment group.

Figure 15 shows the level of IgG in ileal digesta on Day 0 for the triple dose treatment group.

Figure 16 shows the level of IgG in ileal digesta on Day 1 for the single dose treatment group.

Figure 17 shows the level of IgG in ileal digesta on Day 1 for the triple dose treatment group.

Figure 18 shows the level of IgG in ileal digesta on Day 2 for the single dose treatment group.

Figure 19 shows the level of IgG in ileal digesta on Day 2 for the triple dose treatment group.

Figure 20 shows the three-day average of the levels of IgG in ileal digesta.

Figure 21 shows a ribbon diagram of the X-ray crystal structure of human CH3 (DeLano et al., *Science*, 287:1279-1283, 2000). Residue positions that were identified as sites for optimization based on residue frequency analysis are displayed in stick format. The diagram was rendered using MolMol (Koradi et al., *J. Mol. Graphs*, 14:51-55, 1996).

Figure 22 shows sequence alignments of the Fc region of IgG. A. Fragment of the 36 sequence dataset used for residue position frequency analysis and entropy calculations. B. Alignment of the optimal, bovine, human and murine CH3 sequences. Residue positions within the bovine CH3 domain where potentially stabilizing mutations were made are displayed in red. Below the alignments, . = conservative variation; : = strict variation; * = no variation.

Figure 23 shows separation/purification of bCH3. A. Reverse phase HPLC purification of native bCH3 using an Absorbance detector set to 280 nm. The peak eluting at 22.9 minutes is oxidized CH3 while the peak eluting at 25.4 minutes is reduced CH3. B. 10% Polyacrylamide gel (Invitrogen) analysis of purified CH3 constructs.

Figure 24 shows structure and stability analyses of bCH3, mCH3 and hCH3. A. CD spectra of bCH3, mCH3 and hCH3 at 5 °C, pH 7.5. B. Temperature denaturation of bCH3, mCH3 and hCH3 monitored by the far UV CD signal at 217 nm. Curves were fitted to a two-state unfolding model in order to obtain accurate T_m values.

Figure 25 structure and stability analyses of bCH3 and the 6 mutant constructs of the domain. A. CD spectra of native and mutant bCH3 constructs at 5 °C, pH 7.5. B. Plots of the unfolded fractions of each bCH3 construct between 325 and 375 K.

Figure 26 shows a Western blot analysis of callus samples from 8 independent transgenic callus lines. Forty μg of total soluble proteins were loaded per lane. Lanes 1-2: extract from rice callus transformed with pTMR147; Lanes 3-4: extract from rice

callus transformed with pTMR149; Lanes 5-6: extract from rice callus transformed with pTMR156; Lanes 7-8: extract from rice callus transformed with pTMR160; Lane NT: extract from non-transgenic rice callus; Lane MW: Molecular Weight Marker (kD); Lane C1 and C2: 10 and 5 ng of anti-K99 antibody purified from hybridoma cells. GelA: Bis-Tris 4-12% reducing SDS-PAGE; detection with the rabbit anti-mouse H+L (1:500) and the alkaline-phosphatase goat anti-rabbit H+L (1:4000). Gel B: 3-8% (w/v) Tris-acetate NuPAGE non-reducing conditions; detection with rabbit anti-mouse H+L (1:500) followed by the alkaline-phosphatase goat anti rabbit H+L (1:4000). Gel C: 3-8% (w/v) Tris-acetate NuPAGE non-reducing conditions; detection with alkaline-phosphatase anti-mouse Fc (1:2000). Gel D: 3-8% (w/v) Tris-acetate NuPAGE non-reducing conditions; detection with rat anti-mouse kappa (1:200) followed by alkaline-phosphatase goat anti-rat H+L (1:5000).

Figure 27 shows a Western blot analysis of corn seed samples from 6 independent lines. Ten µg of total soluble proteins were loaded per lane. Lanes 1-4: extract from corn seeds transformed with pTMR555 (monoclonal antibody 36/41); Lanes 5-6: extract from corn seeds transformed with pTMR554 (monoclonal antibody 36/41 with ER retention signal); Lane NT: extract from non-transgenic corn seed; Lane MW: Molecular Weight Marker (kD); Lane C1: 40 ng of anti-K88 36/41 antibody purified from hybridoma cells. All gels were 3-8% (w/v) Tris-acetate NuPAGE run in non-reducing conditions. Gel A: detection with alkaline-phosphatase anti-mouse Fc (1:2000). Gel B: detection with rabbit anti-mouse H+L (1:500) followed by the alkaline-phosphatase goat anti rabbit H+L (1:4000). Gel C: detection with rat anti-mouse kappa (1:200) followed by alkaline-phosphatase goat anti-rat H+L (1:5000).

Detailed Description

The following detailed description is provided to aid those skilled in the art in practising the present invention. Even so, this detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present invention.

All publications, patents, patent applications, public databases, public database entries, and other references cited in this application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application,

public database, public database entry, or other reference was specifically and individually indicated to be incorporated by reference.

As used herein "polynucleotide" "oligonucleotide", and "nucleic acid" are used interchangeably and refer to a polymeric (consisting of 2 or more monomers) form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Although nucleotides are usually joined by phosphodiester linkages, the term also includes peptide nucleic acids such as polymeric nucleotides containing neutral amide backbone linkages composed of aminoethyl glycine units (Nielsen et al., *Science*, 254:1497, 1991: Neilsen and Egholm, Peptide Nucleotide Acids: Protocols and Applications, Horizon Scientific Press, Wymondham, Norfolk UK, 1999). This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA as well DNA/RNA hybrids that may be single-stranded, but are more typically double-stranded. In addition, the term also refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all or one or more of the molecules, but more typically involve only a region of some of the molecules. The terms also include known types of modifications, for example, labels, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucelotide modifications such as, for example, those with uncharged linkages (e.g. methyl phosphonates, phophotriesters, phosphoamidates, carbamates etc.), those containing pendant moieties, such as, for example, proteins (including for e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing alkylators, those with modified linkages (e.g. alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide. Polynucleotides include both sense and antisense, or coding and template strands. The terms include naturally occurring and chemically synthesized molecules. Unless specifically limited, the terms encompass nucleic acids containing known analogs of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating

sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucl. Acids Res.*, 19:508 (1991); Ohtsuka et al., *J. Biol Chem.*, 260:2605 (1985); Rossolini et al., *Mol. Cell. Probes*, 8:91 (1994).

As used herein, the term "animal" includes human beings.

By "fragment" or "portion" is meant a full length or less than full length of the nucleic acid sequence encoding, or the amino acid sequence of, a polypeptide or protein. Alternatively, fragments or portions of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments or portions of a nucleotide sequence may range from at least about 6 nucleotides, about 9, about 12 nucleotides, about 15 nucleotides, about 20 nucleotides, about 30 nucleotides, about 50 nucleotides, about 75 nucleotides, about 100 nucleotides or more. By "portion" or "fragment", as it relates to a nucleic acid molecule, sequence or segment of the invention, when it is linked to other sequences for expression, is meant a sequence having at least 80 nucleotides, at least 150 nucleotides, or at least 400 nucleotides. If not employed for expressing, a "portion" or "fragment" means at least about 6, at least about 9, at least about 12, at least about 15, or at least about 20, consecutive nucleotides, e.g., probes and primers (oligonucleotides), corresponding to the nucleotide sequence of the nucleic acid molecules of the invention.

The term "antibody" as used herein includes: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, modified Fab, di Fab, Fab', F(ab')2 or Fv fragment; and a single chain antibody including a light chain or a heavy chain monomer and dimer, as well as a single chain Fv (scFv) in which the heavy and light chain variable domains are joined by a peptide linker.

The term "fragment" as used in relation to an antibody or immunoglobulin molecule includes a Fab, a modified Fab, a di-Fab, a Fab', a $F(ab')_2$ and a Fv fragment.

A "transgene" refers to a gene that has been introduced into the genome by transformation and is stably maintained. Transgenes may include, for example, DNA that is either heterologous or homologous to the DNA of a particular plant to be transformed. Additionally, transgenes may comprise native genes inserted into a non-native organism, or chimeric genes. The term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

The terms "protein," "peptide" and "polypeptide" are used interchangeably herein and mean two or more amino acids linked by peptide bonds.

"DNA shuffling" is a method to introduce mutations or rearrangements, typically randomly, in a DNA molecule or to generate exchanges of DNA sequences between two or more DNA molecules, also typically randomly. The DNA molecule resulting from DNA shuffling is a shuffled DNA molecule that is a non-naturally occurring DNA molecule derived from at least one template DNA molecule. The shuffled DNA preferably encodes a variant polypeptide modified with respect to the polypeptide encoded by the template DNA, and may have an altered biological activity with respect to the polypeptide encoded by the template DNA.

"Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acid sequences that encode identical or essentially identical amino acid sequences, or where the nucleic acid sequence does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance the codons CGT, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations" which are one species of "conservatively modified variations." Every nucleic acid sequence described herein which encodes a polypeptide also describes every possible silent variation, except where otherwise noted. One of skill will recognize that each codon in a nucleic acid (except ATG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

The terms "heterologous DNA sequence," "exogenous DNA segment", "heterologous nucleic acid," each refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of DNA shuffling. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is

foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

"Coding sequence" refers to a DNA or RNA sequence that codes for a specific amino acid sequence and excludes the non-coding sequences 5' and 3' to the coding sequence. It may constitute an "uninterrupted coding sequence", i.e., lacking an intron, such as in a cDNA or it may include one or more introns bounded by appropriate splice junctions, e.g., as may be found in genomic DNA. An "intron" is a sequence of RNA which is contained in the primary transcript but which is removed through cleavage and re-ligation of the RNA within the cell to create the mature mRNA that can be translated into a protein.

The terms "open reading frame" and "ORF" refer to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence. The terms "initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides ("codon") in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation).

"Regulatory sequences" and "suitable regulatory sequences" each refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include enhancers, promoters, translation leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. As is noted above, the term "suitable regulatory sequences" is not limited to promoters. However, some suitable regulatory sequences useful in the present invention will include, but are not limited to constitutive promoters, tissue-specific promoters, development-specific promoters, inducible promoters and viral promoters.

"Promoter" refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. "Promoter" includes a minimal promoter that is a short DNA sequence comprised of a TATA- box and other sequences that serve to specify the site of transcription initiation,

to which regulatory elements are added for control of expression. "Promoter" also refers to a nucleotide sequence that includes a minimal promoter plus regulatory elements that is capable of controlling the expression of a coding sequence or functional RNA. This type of promoter sequence comprises proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions.

"Constitutive expression" refers to expression that occurs throughout a plant and which is not restricted to a specific tissue or location within the plant. Such constitutive expression may use either a constitutive (i.e. permanently switched on) or regulated promoter. "Conditional" and "regulated expression" refer to expression controlled by a regulated promoter, and thus include inducible expression.

"Operably-linked" refers to the association of nucleic acid sequences on single nucleic acid fragment so that the function of one is affected by the other. For example, a regulatory DNA sequence is said to be "operably linked to" or "associated with" a DNA sequence that codes for an RNA or a polypeptide if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation.

"Expression" refers to the transcription or transcription and translation of a polynucleotide, for example an endogenous gene or a transgene, in plants. In the case of antisense constructs, expression may refer to the transcription of the antisense DNA

only. In addition, expression refers to the transcription and stable accumulation of sense (mRNA) or functional RNA. Expression may also refer to the production of protein.

"Partially pure" or "partially purified" means that the substance is free, at least to some extent, from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism (e.g. contaminating proteins, nucleic acids, and/or other biologicals are present in a lesser amount than when the substance is present in the source organism). Purity may be assayed by standard methods, and will ordinarily be at least about 10% pure, at least about 25% pure, at least about 30% pure, at least about 40% pure, at least about 50% pure, at least about 60% pure, or at least about 70% pure. "Substantially pure" or "substantially purified" means at least about 75% pure, at least about 80% pure, at least about 85% pure, at least about 90% pure, at least about 95% pure, at least about 98% pure, or at least 99% pure. The analysis may be weight or molar percentages, evaluated, e.g., by gel staining, spectrophotometry, or terminus labeling etc.

As used herein "isolated polynucleotide" means a polynucleotide that is free of one or both of the nucleotide sequences which flank the polynucleotide in the naturally-occurring genome of the organism from which the polynucleotide is derived. The term includes, for example, a polynucleotide or fragment thereof that is incorporated into a vector or expression cassette; into an autonomously replicating plasmid or virus; into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule independent of other polynucleotides. It also includes a recombinant polynucleotide that is part of a hybrid polynucleotide, for example, one encoding a polypeptide sequence.

The terms "therapeutically-effective" and "effective" when used in conjunction with the term "amount" are intended to qualify the amount of each agent which will achieve the goal of improvement in disorder severity and/or the frequency of incidence over no treatment.

As used herein the phrase "plant material" can refer to a whole plant or a portion of a plant such as the leaves, stems, roots, seeds, and other plant tissue, including organelles such as, for example, plastids, chloroplasts and the like.

Provided herein are isolated polynucleotides mammalian antibodies directed against enterotoxigenic *Escherichia coli* (ETEC) and in particular, ETEC of a strain possessing the K88 or K99 antigen. In one embodiment, the isolated polynucleotide

comprises a sequence encoding the variable region of the heavy chain of an anti-K99 antibody (SEQ ID NO. 3), the heavy chain of an anti-K88 antibody (SEQ ID NOs 11, 13 and 15) or the complements thereof. The sequence may further include the a region coding for the constant region of a heavy chain to form a complete heavy chain antibody fragment, or may contain only the variable region, such as is often seen with single-chain antibodies. Also provided is an isolated polynucleotide encoding a light chain variable region directed against K99 (SEQ ID NO. 4), K88 (SEQ ID NOs 12, 14 and 16) or the complements thereof. The sequence may be incorporated into a larger sequence encoding a complete antibody light chain, that is, containing a light chain constant region in addition to the variable region. Alternatively, the light chain variable region can be incorporated into an antibody lacking constant regions such as single chain (SC) antibodies and single chain Fv (ScFv) antibody fragments.

The isolated polynucleotides disclosed herein can have their sequences altered in order to optimize their expression in a particular host cell or organism (Wada et al., *Nucl Acids Res.*, 18:2367, 1990). For plants see, for example, WO91/16432; Perlak et al., *Proc. Natl. Acad. Sci. USA*, 88:3324, 1991; and Murray et al., *Nucl Acids Res.* 17:477, 1989. In this manner, the genes or gene fragments can be synthesized utilizing plant-preferred codons. See, for example, Campbell and Gowri, *Plant Physiol.*, 92:1, 1990, for a discussion of host-preferred codon usage. Thus, the nucleotide sequences can be optimized for expression in any plant. For example, SEQ ID NO. 1 and SEQ ID NO. 2 represent polynucleotides which encode K99 antibody heavy- and light-chain variable regions, respectively, that have been optimized for expression in plants. Likewise, SEQ ID NOs 5, 7, and 9, represent anti-K88 heavy chain variable regions and SEQ ID NOs 6, 8, and 10 represent anti-K88 light chain variable regions that have been codon optimized for expression in plants. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used. Variant nucleotide sequences and proteins also encompass sequences and protein derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different coding sequences can be manipulated to create a new polypeptide possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. Strategies

– for such DNA shuffling are known in the art. See, for example, Stemmer, *Nature*, 370:389, 1994; Crameri et al., *Nature Biotech.*, 15:436, 1997; Moore et al., *J. Molec. Biol.*, 272:336, 1997; Zhang et al., *Proc. Natl. Acad. Sci. USA*, 94:4504, 1997; Crameri et al., *Nature*, 391:288, 1998; and U.S. Patent Nos. 5,605,793 and 5,837,458.

In one embodiment, antibody sequences are designed to avoid free thiol groups on the surface of the folded antibodies. In another embodiment, the antibodies lack free thiols in the complementary determining regions (CDRs). By free thiol is meant a thiol group that is not involved in disulfide bond formation either within an antibody chain, for example intra light chain bonds, or between chains, for example light chain to heavy chain or heavy chain to heavy chain disulfide bonds. Because of the their location on the surface of the antibody, free thiols are capable of interacting and forming disulfide bonds with molecules other than antibody light and heavy chains. An example of a free thiol is that found in the cysteine residue at position 53 of SEQ ID NO. 18.

Also included are polynucleotides that hybridize to any of SEQ ID Nos. 1-16 or their complements under stringent or very stringent conditions. "Stringent hybridization conditions," "stringent hybridization wash conditions" and "stringent wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. For example, longer sequences hybridize specifically at higher temperatures. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, *Anal. Biochem.*, 138:267, 1984:

$$T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\text{-form}) - 500/L$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. An alternative equation for the calculation of T_m can be found in Sambrook et al. (*Molecular Cloning*, 2nd ed., Cold Spring Harbor Press, 1989) and is:

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%\ G + C) - 0.63(\%\ \text{formamide}) - 600/L$$

where L is the length of the hybrid in base pairs, the concentration of Na^+ is in the range of 0.01M to 0.4M and the G + C content is in the range of 30% to 75%. Equations for hybrids involving RNA can be found in the same reference.

T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, highly stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the above equations, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is common to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" Elsevier, New York, 1993. Generally, stringent hybridization and wash conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH.

Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0. 1X SSC at 60 to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate

stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C.

The present invention also includes polynucleotides that are at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or at least about 99% identical to any one of SEQ ID NOs. 1-16, or their complements. The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches. In one embodiment the comparison window will be of the same length as the reference sequence.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to a specified percentage of residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window, as measured by sequence comparison algorithms or by visual inspection. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent

sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 50%, 60%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

(e)(ii) The term "substantial identity" in the context of a polypeptide indicates that a peptide comprises a sequence with at least 50%, 60%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably 80%, 81%, 82%, 83%, 84%, 85%,

86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, or even more preferably, 95%, 96%, 97%, 98% or 99%, sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, 1970, *supra*. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller, *CABIOS*, 4:11, 1988; the local homology algorithm of Smith et al., *Adv. Appl. Math.*, 2:482, 1981; the homology alignment algorithm of Needleman and Wunsch, *J. Molec. Biol.*, 48:443, 1970; the search-for-similarity-method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444, 1988; the algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA*, 87:2264, 1990, modified as in Karlin and Altschul, *Proc. Natl. Acad. Sci. USA*, 90:5873, 1993.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, CA); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, WI). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al., *Gene*,

73:237, 1988; Higgins et al., *CABIOS*, 5:151, 1989; Corpet et al., *Nucl. Acids Res.*, 16:10881, 1988; Huang et al., *CABIOS*, 8:155, 1992; and Pearson et al., *Meth. Mol. Biol.*, 24:307, 1994. The ALIGN program is based on the algorithm of Myers and Miller, *supra*. The BLAST programs of Altschul et al., *J. Molec. Biol.*, 215:403, 1990; *Nucl. Acids Res.*, 25:3389, 1990, are based on the algorithm of Karlin and Altschul *supra*.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., 1990, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, 1993, *supra*). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is, for example, less than about 0.1, less than about 0.01, or less than about 0.001.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al., *Nuc. Acids Res.*, 25:3389, 1997. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al., *supra*. When utilizing BLAST, Gapped BLAST, or PSI-BLAST, the default parameters of the respective programs (e.g. BLASTN for nucleotide sequences, BLASTX for proteins) can be used. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci.*, 89:10915, 1989). See http://www.ncbi.n1m.nih.gov. Alignment may also be performed manually by inspection.

Comparison of nucleotide sequences for determination of percent sequence identity to the sequences disclosed herein is preferably made using the BlastN program (version 1.4.7 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

Also included are fragments of any one of SEQ ID NO 1-16 or their complements that are at least 15, at least 20, at least 30, at least 50 or at least 100 nucleotides in length. In one embodiment, these fragments comprise a sequence encoding a complementary determining region (CDR) involved in K88 or K99 antigen binding. Complementary determining regions, also known are hypervariable regions, are short segments within the variable region of an antibody that exhibit exceptional variability to create antigen binding sites. The position of the CDRs within a light chain or heavy chain can easily be identified if the Kabat numbering system is applied to the light and heavy chain (Kabat et al. 1987, in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services, NIH, USA).

The present inventive discovery also includes polypeptides encoded by any one of SEQ ID NO. 1-16 and in particular SEQ ID NO. 17, 18 and 67-72. As is well known in the art and discussed herein, the genetic code is degenerate such that more than one

nucleotide sequence can encode the same polypeptide. Thus, within the scope of the present invention are all polynucleotides encoding any one of SEQ ID NOs. 17, 18 and 67-72. Also included are fragments of SEQ ID NOs. 17, 18 and 67-72 that comprise at least one complementary determining region, or a fragment thereof, that interacts and binds to the K88 or K99 antigen of an ETEC, as well as the nucleotide sequences encoding such CDRs or fragments.

Those of ordinary skill in the art are aware that modifications in the amino acid sequence of a peptide, polypeptide, or protein can result in equivalent, or possibly improved, second generation peptides, etc., that display equivalent or superior functional characteristics when compared to the original amino acid sequence. The present invention accordingly encompasses such modified amino acid sequences. Alterations can include amino acid insertions, deletions, substitutions, truncations, fusions, shuffling of subunit sequences, and the like, provided that the peptide sequences produced by such modifications have substantially the same functional properties (e.g. binding specificity, at least the same or greater binding affinity, at least the same or greater half-life) as the naturally occurring counterpart sequences disclosed herein

One factor that can be considered in making such changes is the hydropathic index of amino acids. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein has been discussed by Kyte and Doolittle (*J. Mol. Biol.*, 157: 105-132, 1982). It is accepted that the relative hydropathic character of amino acids contributes to the secondary structure of the resultant protein. This, in turn, affects the interaction of the protein with molecules such as enzymes, substrates, receptors, DNA, antibodies, antigens, etc.

Based on its hydrophobicity and charge characteristics, each amino acid has been assigned a hydropathic index as follows: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

As is known in the art, certain amino acids in a peptide or protein can be substituted for other amino acids having a similar hydropathic index or score and produce a resultant peptide or protein having similar biological activity, i.e., which still

retains biological functionality. In making such changes, it is preferable that amino acids having hydropathic indices within ±2 are substituted for one another. More preferred substitutions are those wherein the amino acids have hydropathic indices within ±1. Most preferred substitutions are those wherein the amino acids have hydropathic indices within ±0.5.

Like amino acids can also be substituted on the basis of hydrophilicity. U.S. Patent No. 4,554,101 discloses that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0 ±1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ±1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). Thus, one amino acid in a peptide, polypeptide, or protein can be substituted by another amino acid having a similar hydrophilicity score and still produce a resultant protein having similar biological activity, i.e., still retaining correct biological function. In making such changes, amino acids having hydropathic indices within ±2 are preferably substituted for one another, those within ±1 are more preferred, and those within ±0.5 are most preferred.

As outlined above, amino acid substitutions in the peptides of the present invention can be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, etc. Exemplary substitutions that take various of the foregoing characteristics into consideration in order to produce conservative amino acid changes resulting in silent changes within the present peptides, etc., can be selected from other members of the class to which the naturally occurring amino acid belongs. Amino acids can be divided into the following four groups: (1) acidic amino acids; (2) basic amino acids; (3) neutral polar amino acids; and (4) neutral non-polar amino acids. Representative amino acids within these various groups include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cysteine, cystine, tyrosine, asparagine, and glutamine; and (4) neutral non-polar amino acids such as alanine, leucine, isoleucine,

valine, proline, phenylalanine, tryptophan, and methionine. It should be noted that changes which are not expected to be advantageous can also be useful if these result in the production of functional sequences.

Also included are vectors containing SEQ ID NOs. 1-16 or any combination thereof. "Vector" is defined to include, *inter alia*, any plasmid, cosmid, phage or binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular or plastid genome, or exist extrachromosomally (e.g., autonomous replicating plasmid with an origin of replication). Specifically included are shuttle vectors by which is meant a vector capable, naturally or by design, of replication in two different host organisms which may be selected from bacteria, plant, mammalian, yeast or fungal cells. Also included are cloning vectors which typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance, hygromycin resistance or ampicillin resistance. The term "vector" also includes expression vectors. An expression vector is a cloning vector designed so that a coding sequence inserted at a particular site will be transcribed, or transcribed and translated into a protein.

The present invention additionally provides expression cassettes comprising SEQ ID NOs. 1-16 or any combination thereof. "Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. The expression of the nucleotide

sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, the promoter can also be specific to a particular tissue or organ or stage of development.

Such expression cassettes usually comprise a transcriptional initiation region linked to a nucleotide sequence of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

An expression cassette will typically include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. For expression in plants, convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau et al., *Mol. Gen. Genetics*, 262:141, 1991; Proudfoot, *Cell*, 64:671, 1991; Sanfacon et al., *Genes Dev.*, 5:141, 1991; Mogen et al., *Plant Cell*, 2:1261, 1990; Munroe et al., *Gene*, 91:151, 1990; Ballas et al., *Nucl. Acids Res.*, 17:7891, 1989; Joshi et al., *Nucl. Acids Res.*, 15:9827, 1987.

The isolated polynucleotides of the present invention can be incorporated into cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e., not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. Where it is desired to introduce both heavy and light chains (or fragments thereof e.g. the variable or constant regions) into an expression system, the nucleotide sequences encoding said heavy and light chain components may be cloned into the same vector, or alternatively the light chain (or fragment thereof) may be cloned into a first vector and the heavy chain component (or fragment thereof) may be cloned into a second vector. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences. U.S. Patent No. 4,237,224, describes the production of expression systems in the form of recombinant plasmids

using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eukaryotic cells grown in culture. Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gtWEST.B, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYCl84, pUC8, pUC9, pUCl8, pUCl9, pLG339, pR290, pKC37, pKC1O1, SV40, pBluescript Il SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif), pQE, pIH82l, pGEX, pET series (see Studier et. al., "Gene Expression Technology", in *Methods in Enzymology*, 185:60-89, 1990), and any derivatives thereof. Suitable vectors are continually being developed and identified. Recombinant molecules can be introduced into cells via transformation, transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al. or Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York (1982 or 1989, respectively). A variety of host-vector systems may be utilized to express the antibody or antibody fragments encoded by the polynucleotides provided herein. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria viruses or transformed via particle bombardment (i.e., biolistics). The use of plants and plant cells, in conjunction with suitable vectors, as suitable expression systems is particularly preferred.

The expression elements of the vectors used in the systems described above vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used. Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA, "mRNA" translation). Transcription of

DNA is dependent upon the presence of a promoter. Thus, in certain embodiments the nucleotide sequences of the present invention, and in particular SEQ ID NOs. 1-16 are operably linked to a promoter. The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system, and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells. Similarly, translation of DNA in prokaryotes depends upon the presence of the proper prokaryotic signals which differ from those of eukaryotes. Efficient translation of DNA in prokaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 165, rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Koberts and Lauer, *Methods in Enzymology* 68:473, 1979.

The choice of promoter will vary depending on the temporal and spatial requirements for expression, the target species, and the level of expression desired. In one embodiment expression of the nucleotide sequences is driven by promoters shown to be functional in plants. Expression under more than one type of condition may be desired, and thus expression in multiple tissues and/or at multiple developmental stages may be desirable. Promoters from dicotyledons have been shown to be operational in monocotyledons and *vice versa*. There is no restriction on the choice of promoter, as the principal selection criterion is that they be operational in driving the expression of the nucleotide sequences in the desired cell.

Suitable promoters that are expressed constitutively include the CaMV 35S and 19S promoters, as well as promoters from genes encoding actin or ubiquitin. The ubiquitin promoter is particularly suitable. A typical constitutive promoter of plant origin is the promoter of the cowpea trypsin inhibitor gene. However, plant promoters need not be of plant origin. For example, promoters derived from plant viruses, such as the CaMV 35S promoter and the Cestrum promoter (referred to herein as CMPS promoter) from Cestrum Yellow Leaf Curling Virus, or from *Agrobacterium tumefaciens*, such as the T-DNA promoters, can be plant promoters, which are suitable

for use in the invention. The CMPS promoter is particularly suitable (see published International Patent Publication No. WO01/73087).

It should be noted that the sequences of the invention encoding light and heavy chains (or fragments thereof) may each be placed under the control of a promoter of different strength. In one embodiment, sequences encoding light chains are operably linked to promoters that are strong relative to the promoters operably linked to the sequences encoding heavy chains, thus encouraging a higher level of expression of light chain in comparison to the level of heavy chain. Alternatively, the sequences of the invention encoding light and heavy chains (or fragments thereof) may be placed under the control of the same promoter or under different promoters of similar strength. Examples of constructs containing promoters of different strengths driving expression of sequences encoding light and heavy chains can be found in Table 1.

In one embodiment, specific promoters are used. By "specific promoters" is meant promoters that have a high preference for driving gene expression in the specified tissue and/or at the specified time during the concerned tissue or organ development. By "high preference" what is meant are at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold increase in expression in the desired tissue over the expression in any undesired tissue.

The nucleotide sequences of this invention can also be expressed under the regulation of promoters that are chemically regulated. This enables the present sequences to be synthesized only when the plants are treated with inducing chemicals. Preferred technology for chemical induction of gene expression is detailed in the published application EP 0 332 104 and US Patent No. 5,614,395. A typical example of an inducible promoter, which can be utilized with the polynucleotides of the present invention, is PARSKI, the promoter from the Arabidopsis gene encoding a serine-threonine kinase enzyme, and which is induced by dehydration, abscissic acid and sodium chloride (Wang and Goodman, *Plant J.* 8:37, 1995). Another suitable promoter for chemical induction is the tobacco PR-1a promoter. Still another chemically inducible promoter is induced by glucocorticoids, which are not naturally found in plants, and is disclosed by Chua in US Patent No. 6,063,985.

Another class of suitable promoters is tissue specific promoters. Non-limiting examples of tissue specific promoters include green tissue specific, root specific, stem specific, and flower specific. Promoters suitable for expression in green tissue include

many that regulate genes involved in photosynthesis many of which have been cloned from both monocotyledons and dicotyledons. A suitable promoter is the maize PEPC promoter from the phosphoenol carboxylase gene (Hudspeth & Grula, *Plant Molec Biol* 12:579, 1989). A suitable promoter for root specific expression is that described by de Framond (*FEBS* 290:103, 1991) and in EP 0 452 269. An illustrative stem specific promoter is that described in US Patent No. 5,625,136 which drives expression of the maize *trpA* gene. Tissue specific expression can be found in epidermis, root, vascular tissue, meristem, cambium, cortex, pith, leaf, and flower of plants transformed according to the present invention, where a suitable tissue-specific promoter is selected and an expression construct is created using methods known in the art.

One embodiment of the invention encompasses transgenic plants expressing at least one of the nucleotide sequences of the invention in a seed-preferred or seed-specific fashion, either constitutively or induceably. A particularly preferred embodiment provides seed specific expression of at least one sequence of the present invention, wherein each polynucleotide is operably linked with a signal peptide and a vacuolar and/or endoplasmic reticulum targeting signal for optimum expression and desired localization. Further embodiments include transgenic plants expressing the nucleotide sequences in green tissue either constitutively or following induction.

Examples of useful tissue specific, developmentally regulated promoters include fruit-specific promoters such as the E4 promoter (Cordes et al., *Plant Cell* 1:1025, 1989), the E8 promoter (Deikman et al., *EMBO J.*, 7: 3315, 1988), the kiwifruit actinidin promoter (Lin et al., *Proc. Natl. Acad. Sci. USA*, 90: 5939, 1993), the 2A11 promoter (Houck et al., U.S. Patent 4,943,674), and the tomato pZ130 promoter (U.S. Patents 5,175, 095 and 5,530,185); the β-conglycinin 7S promoter (Doyle et al., *J. Biol. Chem.*, 261: 9228, 1986; Slighton and Beachy, *Planta* 172: 356, 1987), and seed-specific promoters (Knutzon et al., *Proc. Natl. Acad. Sci. USA* 89: 2624, 1992; Bustos et al., *EMBO J.* 10: 1469, 1991; Lam and Chua, *J. Biol. Chem.* 266: 17131, 1991; Stayton et al., *Aust. J. Plant. Physiol.* 18: 507, 1991). Fruit-specific gene regulation is discussed in U.S. Patent 5,753,475. Other useful seed-specific promoters include, but are not limited to, the napin, phaseolin, zein, soybean trypsin inhibitor, 7S, ADR12, ACP, stearoyl-ACP desaturase, oleosin, *Lasquerella* hydroxylase, and barley aldose reductase promoters (Bartels, *Plant J.* 7: 809, 1995), the EA9 promoter (U.S. Patent 5,420,034), and the Bce4 promoter (U.S. Patent 5,530,194). Useful embryo-

specific promoters include the corn globulin 1 and oleosin promoters. Useful endosperm-specific promoters include the rice glutelin-1 promoter, the promoters for the low-pI α-amylase gene (Amy32b) (Rogers et al., *J. Biol. Chem.* 259: 12234, 1984), the high-pI α-amylase gene (Amy 64) (Khurseed et al., *J. Biol. Chem.* 263: 18953, 1988), and the promoter for a barley thiol protease gene ("Aleurain") (Whittier et al., *Nucleic Acids Res.* 15: 2515, 1987). Plant functional promoters useful for preferential expression in seed plastids include those from plant storage protein genes and from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl et al., *Seed Sci. Res.* 1: 209, 1991), phaseolin, zein, soybean trypsin inhibitor, ACP, stearoyl-ACP desaturase, and oleosin. Seed-specific gene regulation is discussed in EP 0 255 378 B1 and U.S. Patents 5,420,034 and 5,608,152 . Promoter hybrids can also be constructed to enhance transcriptional activity (Hoffman, U.S. Patent No. 5,106,739), or to combine desired transcriptional activity and tissue specificity.

In addition to the selection of a suitable promoter, constructions for expression of the sequences of the present invention in plants may also include an appropriate transcription terminator attached downstream of the heterologous nucleotide sequence. Several such terminators are available and known in the art, such as *tml* from CaMV, E9 from *rbcS*. Any available terminator known to function in plants can be used in the context of this invention.

Numerous other sequences can be incorporated into expression cassettes described in this invention. These include sequences that have been shown to enhance expression such as intron sequences from *Adh1* and *bronze1*, and viral leader sequences, such as those from TMV, MCMV and AMV, in conjunction with the present invention. The expression vectors of the present invention may further include intronic portions of the PAT1 gene, as described in U.S. Patent No. 5,681, 277 to Rose and Last, which discloses that inclusion of at least one of the first two introns of the PAT1 gene causes increased expression of the polypeptide of interest.

It may be desired to target expression of the nucleotide sequences of the present invention to different cellular localizations in the plant. In some cases, localization in the cytosol may be desirable, whereas in other cases, localization in some subcellular organelle may be preferred. Subcellular localization of expression is undertaken using techniques well known in the art. Typically, the DNA encoding the target peptide from

a known organelle-targeted gene product is manipulated and fused upstream of the nucleotide sequence. For example, most nuclear-encoded mitochondrial proteins are imported into the mitochondria due to the presence of a targeting peptide, the mitochondrial leader peptide (Neupert, *Ann Rev Biochem*, 66:863, 1997). Typically, leader peptides are proteolyzed after the attached protein has been imported into a mitochondrion. Mitochondrial leader peptides can be joined to non-mitochondrial proteins to target the resultant recombinant passenger protein into the mitochondria. A nucleic acid sequence encoding a mitochondrial leader peptide may be obtained through genetic engineering from an existing mitochondrially-targeted protein, for instance N. plumbaginifolia ATPase-beta. Alternatively, the coding sequence for a mitochondrial leader peptide may be engineered in the laboratory, for instance through oligonucleotide synthesis. Additionally, it has been demonstrated that tandem duplication of mitochondrial leader peptides can enhance the targeting of some proteins to mitochondria. (Galanis *et al., FEBS Lett* 282:425 1991). It therefore may be useful in some embodiments of this invention to use multiple, tandem copies of a chosen mitochondrial leader sequence in making the transformation construct. For the purpose of the current invention, mitochondrial leader peptides are quantitatively characterized by the ability to target at least 25% of the stable recombinant protein into the mitochondria. Such mitochondrial leader peptides will more preferably target at least 50% and still more preferably at least 75% of the stable recombinant protein into the mitochondria. The amount of mitochondrially-targeted recombinant protein can be measured by cellular fractionation followed by, for example, quantitative immunoblot analysis using a antiserum specific to the recombinant protein.

Many such target sequences are also known for the chloroplast and their functioning in heterologous constructions has been shown (See, for example, US Patent No. 6,388,168 B1; Ruf et al., *Nature Biotech.*, 19:870, 2001; Heifetz, *Biochimie*, 82:655, 2000; Heifetz and Tuttle, *Curr. Opin. Plant Biol.*, 4:157, 2001). In various embodiments, the expression of the sequences of the present invention is targeted to the mitochondrion, chloroplast, chromoplast, amyloplast, endoplasmic reticulum, or to the vacuoles of the host cells. In one embodiment, the expression is targeted to protein bodies in seeds or storage tissue. Techniques to achieve this are well known in the art.

The sequences of the present invention are used to produce in plants antibodies or antibody fragments directed against enteric pathogens and in particular ETEC. Thus

in one aspect of the invention nucleotide sequences or vectors of the invention are introduced into a plant cell using appropriate methodology. Whole plants, which are capable of expressing the antibodies or antibody fragments of the invention, are then regenerated from such transformed cells, and said antibodies or antibody fragments are expressed. Optionally said antibody or antibody fragments are purified from the plants/plant material in which they are expressed, using standard purification procedures. The skilled man will appreciate that where it is desired that both heavy and light chains (or fragments thereof) are to be co-expressed in the same plant, such co-expression can be achieved in a number of ways. For example: (i) by introducing a single vector encoding both heavy and light chains (or fragments thereof) into the plant cell; (ii) by introducing the different chains (or fragments thereof) on different vectors into the same plant cell; or (iii) transforming a vector encoding the light chain (or fragment thereof) into a first plant cell, transforming a vector encoding the heavy chain (or fragment thereof) into a second plant cell, regenerating plants from said transformed cells and crossing said plants to obtain progeny expressing both the light and heavy chains (or fragments thereof).

The antibodies/antibody fragments that are produced in plants may be of any of the known classes of antibodies such as IgG, IgA, IgM and IgE.

The present invention also encompasses the use of sequences encoding neutralizing monoclonal antibodies. The production of monoclonal antibodies is routine in the art. Methods for the production of monoclonal antibodies can be found in standard texts such as Ausubel et al., *Short Protocols in Molecular Biology*, 2nd ed, Wiley & Sons, 1992; King, *Applications and Engineering of Monoclonal Antibodies*, Taylor & Francis, 1999; Zola, *Monoclonal Antibodies*, Springer Verlag, 2000. Production of monoclonal antibodies against ETEC can be found, for example, in US Patent No. 4,443,549.

Briefly, an animal is administered an antigen of interest from an ETEC using standard methods well known in the art. Once the animal has mounted an immune response, antibody secreting cells are isolated, typically from spleen cells, but other sources of antibody secreting B cells such as blood can be used. Once obtained, the cells are immortalized commonly by fusion to a myeloma cell line or by viral transformation such as by Epstein-Barr virus transformation. The immortalized (hybridoma) cells are then selected, grown and tested for secretion of antibodies

directed against the antigen of interest. Once suitable cells are identified, they are clonally expanded and the clonal cells are tested for antibody production. Monoclonal antibodies used in the practice of the present invention can be homologous, that is derived from antibody secreting cells from an animal of the same species as the animal to which they are administered or heterologous, that is derived from cells of a different species. In one embodiment, the monoclonal antibodies used are from a mouse monoclonal cell line. In another embodiment, the monoclonal antibodies used are derived from bovine antibody secreting B cells. Once the appropriate antibody secreting cells have been identified, the polynucleotide sequences encoding the antibodies can be isolated without undue experimentation using standard molecular biology techniques known in the art and described herein.

The present invention also encompasses the use of recombinant antibodies. One method for the production of recombinant antibodies is by the phage display method. Methods for the production and selection of antibodies using phage display are well known in the art and can be found, for example in Vaughan et al., *Nature Biotech.* 16:535-539, 1998; Watkins and Ouwehand, *Vox Sanguinis* 78:72-79, 2000; and the references cited therein.

Antibody production by phage display involves the generation of combinatorial libraries of immunoglobulin variable heavy chain (VH) and variable light chain (VL) sequences. These sequences are inserted into phage genes encoding coat proteins so that the VH and VL sequences are expressed (displayed) on the coat of filamentous bacteriophage. Phage expressing VH and VL regions of interest are selected by an affinity selection process commonly referred to as panning.

VH and VL sequences are generated by isolating mRNA from antibody secreting B-cells and amplifying the mRNA by RT-PCR using primers to conserved regions of the immunoglobulin gene. mRNA can be obtained from B-cells obtained directly from an animal, preferably an animal immunized with the antigen of interest, or from hybridoma cells producing antibodies against the antigen of interest. Once obtained, the VH and VL cDNA can be recombined by sequential cloning of VH and VL sequences into the same vector (Huse et al., *Science,* 246:1275-1281, 1989), by combinatorial infection using the *lox*Cre site-specific recombination system of bacteriophage P1 (Waterhouse et al., *Nuc. Acids Res.,* 21:2265-2266, 1993), or by PCR assembly (Clackson et al., *Nature,* 352:624-628, 1991; Marks et al., *J. Molec. Biol.,*

222:581-597, 1991). Alternatively, synthetic repertories of variable region sequences can be used as described, for example, in Griffiths et al. (*EMBO J.*, 13:3245-3260, 1994).

Once a phage display library has been constructed, phage displaying reactive antibodies are selected by panning. Typically, purified antigen is attached to a solid substrate such as a plastic surface or an affinity chromatography column. The antigen may be attached to the surface directly or through an intermediary such as the streptavidin/biotin system. Phages to be selected are incubated with the antigen and non-binding phage washed away. A single round of selection can enrich for specific phage by 20 to 1,000 fold. Typically, several rounds of selection are carried out to increase specificity and affinity. Once phage displaying antibodies of the desired characteristics are identified, they are grown in a suitable bacterial host, the DNA encoding the antibody is isolated, and the DNA can then be sequenced. The polynucleotides can then be used to transform plants using any standard technique including those described herein.

Alternatively, chimeric antibodies can be used. Chimeric antibodies are those in which different regions of the immunoglobuin molecule are from different sources. Typically, chimeric antibodies comprise a mouse variable region and a constant region derived from the species to which the antibody is to be administered, for example a mouse variable region and a bovine constant region. Production of chimeric antibodies has become routine in the art and does not require any in depth structural knowledge of the antibody-antigen interaction (Watkins and Ouwehand, *Vox Sanguinis*, 78:72-79, 2000). Another form of chimeric antibody can be produced by the process known as "CDR grafting" (Jones et al., *Nature*, 321:522-525, 1986). CDRs (complementarity determining regions) are apical loops between the anti-parallel β-pleated sheets of a structure known as the immunoglobulin fold. The β-pleated sheets form a framework to correctly orientate the CDRs for interaction with the antigen. In CDR grafting, the CDRs of a specific antibody from one species (typically murine, but also potentially chicken, goat, ovine, bovine, CDRs) are grafted onto an appropriate β-pleated sheet framework (typically a human framework).

Additionally, antibodies can be obtained from transgenic animals and in particular transgenic mice (Bruggemann and Taussig, *Curr. Opin. Biotechnol.*, 8:455-458, 1997). In this method, the endogenous mouse immunoglobulin genes are

inactivated and replaced with unrearranged immunoglobulin sequences from the species of interest. Monoclonal antibodies of the species of interest are then produced from the transgenic mice using the methods described above. Once hybridoma lines producing suitable monoclonal antibodies are identified, the polynucleotides encoding the antibodies can be isolated and transformed into plants.

The sequences of the present invention may also be used to produce single chain Fv antibodies(scFv) and in particular scFv antibodies directed against ETEC antigens. scFv Antibodies typically comprise a heavy chain variable region and a light chain variable region joined together by a linker. Thus, scFv antibodies lack the constant regions found in naturally occurring antibodies. The linker is typically a polypeptide, but other linkers such as chemical linkers can be used. The linker is typically of a length to allow proper orientation of the scFv to allow the light and heavy chain variable regions to interact with the antigen. In some instances the linker will be a cleavable linker. Methods of the production and use of scFv antibodies are well known in the art and can be found, for example, in US Patent Nos. 4,947,778; 5,260,203 and 5,863,765.

Alternatively the sequences disclosed herein can be used to produce antibodies lacking a light chain, especially those directed against ETEC and in particular antibodies directed against ETEC that lack a light chain and are suitable for production in plants. Antibodies lacking a light chain are naturally produced by animals of the family *Camelidae*. These antibodies comprise a dimer of two heavy chains, but lack the light chain associated with antibodies in most mammals. In addition, the constant region of the heavy chain typically lacks a CH1 domain. Detailed information regarding these antibodies can be found in numerous references, for example, US Patent Nos. 5,759,808; 5,800,988; 5,840,526; 5,874,541; 6,005,079; 6,015,695 and European Patent Office publication EP 1118669 A2. Also included are antibody fragments of such antibodies comprising only the variable region known in the art as VHH antibodies, as well as chimeric antibodies comprising a Camelid variable region and heterologous constant region, for example a bovine constant region. Methods for the production of chimeric antibodies are well known in the art. For example, vectors can be produced in which a nucleotide sequence encoding a Camelid-type heavy chain variable region is spliced in frame to a sequence encoding a constant region from a non-Camelid species.

Additionally, sequences encoding different variable regions can be combined to produce a single antibody with variable regions directed to different antigens or epitopes. For example, a chimeric IgG antibody can be produced with a first variable region directed to a K88 antigen and a second variable region directed to a K99 antigen. Alternatively the plant can be transformed with constructs encoding antibodies that when assembled possess variable regions directed to different epitopes of the same antigen.

The antibody may also incorporate a purification moiety. The purification moiety may or may not be cleaved from the antibody during or after purification. A non-limiting example of a purification moiety is the poly-histidine nickel-chelating amino acid sequence described in US Patent No. 5,594,115. Another example is an epitope used for purification such as is described in US Patent No. 6,379,903.

In one embodiment, at least one of the sequences disclosed herein is introduced into a plant or plant cell for expression. The polynucleotides described herein can be introduced into the plant cell in a number of ways known to those of ordinary skill in the art. Those skilled in the art will appreciate that the choice of method might depend on the type of plant targeted for transformation. Suitable methods of transforming plant cells include, but are not limited to, microinjection (Crossway et al., *BioTechniques* 4:320, 1986), electroporation (Riggs et al., *Proc Natl Acad Sci USA* 83:5602, 1986), *Agrobacterium*-mediated transformation (Hinchee et al., *Biotechnology* 6:915, 1988; Ishida et al. *Nature Biotechnology* 14:745, 1996, direct gene transfer (Paszkowski et al., *EMBO J.*, 3:2717, 1984; Hayashimoto et al., *Plant Physiol.*, 93:857, 1990 (rice), and ballistic particle acceleration using devices. Specific examples can be found in Sanford et al., U.S. Patent 4,945,050; and McCabe et al., *Biotechnology* 6:923, 1988; Weissinger et al., *Annual Rev Genet.*, 22:421, 1988; Sanford et al., *Particulate Science and Technology* 5:27, 1987 (onion); Svab et al., *Proc Natl Acad Sci US*, 87:8526, 1990 (tobacco chloroplast); Christou et al. *Plant Physiol.*, 87:671, 1988 (soybean); McCabe et al., *Bio/Technology* 6:923, 1988 (soybean); Klein et al., *Proc Natl Acad Sci USA*, 85:4305, 1988 (maize); Klein et al., *Bio/Technology*, 6:559, 1988 (maize); Klein et al., *Plant Physiol.*, 91:440, 1988 (maize); Fromm et al., *Bio/Technology* 8:833, 1990; Gordon-Kamm et al., *Plant Cell* 2:603, 1990 (maize); Koziel et al., *Biotechnology* 11:194, 1993 (maize)); Shimamoto et al., *Nature* 338:274, 1989 (rice); Christou et al., *Biotechnology* 9: 957, 1991 (rice); Datta et al., *Bio/Technology* 8:736, 1990 (rice);

European Patent Application EP 0 332 581 (orchard grass and other *Pooideae*); Vasil et al., *Biotechnology* 11:1553, 1993 (wheat); Weeks et al., *Plant Physio.*, 102:1077, 1993 (wheat); Wan et al., *Plant Physiol.*, 104:37, 1994 (barley); Jahne et al., *Theor. Appl. Genet.*, 89:525, 1994 (barley); Umbeck et al., *Bio/Technology* 5:263, 1987 (cotton); Casas et al., *Proc. Natl. Acad. Sci. USA* 90:11212, 1993 (sorghum); Somers et al.. *Bio/Technology* 10:1589, 1992 (oat); Torbert et al., *Plant Cell Reports* 14:635, 1995 (oat); Weeks et al., *Plant Physiol* 102:1077, 1993 (wheat); Chang et al., WO 94/13822 (wheat) and Nehra et al., *The Plant Journal* 5:285, 1994 (wheat).

Suitable exemplary methods for the introduction of recombinant DNA molecules into maize by microprojectile bombardment can be found in Koziel et al., *Biotechnology* 11:194, 1993); Hill et al., *Euphytica* 85:119, 1995); and Koziel *et al.* (*Annals New York Acad. Sci.*, 792:164, 1996). An additional embodiment uses the protoplast transformation method for maize as disclosed in EP 0 292 435. Transformation of plants can be undertaken with a single polynucleotide of the present invention or multiple polynucleotides, for example by co-transformation, and both these techniques are suitable for use with the sequences described herein.

In another embodiment, at least one nucleotide sequence of the present invention is directly transformed into the plastid genome. Plastid transformation technology is extensively described in U.S. Patent Nos. 5,451,513, 5,545,817, 5,545,818, and 6,388,168 B1, in PCT Publication No. WO 95/16783, and in McBride et al., *Proc. Natl. Acad. Sci. USA*, 91:7301, 1994). The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, using biolistics or protoplast transformation, including calcium chloride or PEG-mediated transformation. The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Typically, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab et al., *Proc. Natl. Acad. Sci. USA*, 87:8526, 1990; Staub and Maliga, Plant Cell, 4:39, 1992). This results in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allows creation of a plastid-targeting vector for introduction of foreign genes (Staub and Maliga, *EMBO J.*

12:601, 1993). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial *aadA* gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3'-adenyltransferase (Svab and Maliga, *Proc Natl Acad Sci USA,* 90:913, 1993). Previously, this marker had been used successfully for high-frequency transformation of the plastid genome of the green alga *Chlamydomonas reinhardtii* (Goldschmidt-Clermont, *Nucl. Acids Res.*, 19:4083, 1991). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the invention.

Typically, approximately 15-20 cell division cycles following transformation are required to reach a homoplastidic state. Plastid expression, in which genes are inserted by homologous recombination into all of the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In one embodiment, a polynucleotide sequence of the present invention is inserted into a plastid-targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplastic for plastid genomes containing a nucleotide sequence of the present invention are obtained, and are preferentially capable of high expression of the nucleotide sequence.

Once a polynucleotide of the invention has been transformed into a particular plant species or variety, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques. For example, in order to maximize antibody production, the polynucleotides can be moved into high protein varieties, for example, varieties that have high levels of protein production in their seeds or other, preferably edible, parts. Alternatively, plant lines which have been shown to exhibit an increased ability to assemble complex proteins can be used. Particularly useful plants include the agronomically important crops, for example, corn, rice, alfalfa and soybeans. The genetic properties introduced into the transgenic seeds and plants described can be passed on by sexual reproduction and can thus be maintained and propagated in progeny plants. Thus, included within the scope of the invention are hybrid plants or seeds produced using any of the plants of the present invention.

Alternately, genetic properties introduced into transgenic plants as described above are passed on through apomictic breeding programs. Apomixis is the naturally occurring ability of some plant species to reproduce asexually through seeds, with the result that apomictically produced seeds inherit their genes exclusively from the mother. During apomixis, the reduction in chromosome number used to produce haploid gametes is bypassed, such that an unreduced reproductive cell (unreduced egg) with unchanged chromosome constitution is produced that ultimately develops into an embryo without fertilization, in a process called parthenogenesis. Apomixis occurs in over 400 plant species in a variety of plant families, and is genetically controlled. *Tripsacum*, preferably *Tripsacum dactyloides*, an apomictic wild relative of wheat, is a source of genetic resources for introgressing apomixis into cultivated crops such as wheat (Hoisington et al., *Proc. Natl. Acad. Sci.*, 96:5937, 1999).

Embodiments of the present invention also relate to a transgenic plant cell, tissue, organ, seed or plant part obtained from the transgenic plant. Also included within the invention are transgenic descendants of the plant as well as transgenic plant cells, tissues, organs, seeds and plant parts obtained from the descendants.

In one embodiment, the polynucleotides of the present invention have been optimized for expression in plants, but encode the same amino acid sequence as any one of SEQ ID NOs. 1-16 or SEQ ID NOs 17, 18, and 67-72. Although in many cases heterologous genes can be expressed in plants at high levels without modification, low expression in transgenic plants may result from nucleotide sequences having codons that are not preferred in the transgenic plants. It is known in the art that all organisms have specific preferences for codon usage, and the codons of the nucleotide sequences described in this invention can be changed to conform with particular plant preferences, while maintaining the amino acids encoded thereby. Furthermore, high expression in plants is best achieved from coding sequences that have at least about 35% GC content, often more than about 45%, typically more than about 50%, and more typically more than about 60% GC content. Nucleotide sequences, which have low GC contents, may express poorly in plants due to the existence of ATTTA motifs, which may destabilize messages, and AATAAA motifs that may cause inappropriate polyadenylation. Although preferred gene sequences may be adequately expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of

monocotyledons or dicotyledons as these preferences have been shown to differ (Murray et al., *Nucl. Acids Res.*, 17:477, 1989). In addition, the nucleotide sequences can be screened for the existence of illegitimate splice sites that may cause message truncation. Changes required to be made within the nucleotide sequences such as those described above can be made using techniques of site directed mutagenesis, PCR, and synthetic gene construction and the methods described in the published patent applications EP 0 385 962, EP 0 359 472 and WO 93/07278.

Using the methods described herein, one skilled in the art can express the polynucleotides of the present invention in a variety of plants. The exact choice of plant will vary with the individual. In one embodiment the plants expressing the polynucleotides described herein are monocots or dicots. Non-limiting examples of plants that can be used in the practice of the invention include, acacia, alfalfa, aneth, apple, apricot, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussel sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, onion, orange, ornamental plants, papaya, parsley, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, watermelon, wheat, yam, and zucchini. In one embodiment the plant is selected from the group consisting of corn, wheat, rye, barley, rice, oats, soybean, and tobacco. In a further embodiment the plant is selected from the group consisting of corn, rice, soybeans, wheat and sugarbeets. Safflower is considered to be a dicotyledenous plant that is particularly suitable for use with the invention.

Plants expressing the sequences described herein can be used without further processing other than standard harvesting and storage techniques. If desired, plants and/or tissue from plants such as green tissue, fruits or seeds, can be further processed, for example, by drying, chopping, grinding, cracking, rolling, extruding, pelleting, defatting, or other methods known in the art.

Polypeptides encoded by polynucletotides described herein and expressed in host cells, for example plants, can be used unpurified, partially purified or in substantially purified form. Methods for purification of proteins are well known to those skilled in the art. These include precipitation, for example ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, high performance liquid chromatography (HPLC), electrophoresis under native or denaturing conditions, isoelectric focusing, and immunoprecipitation. Specific examples of antibody purification can be found in U.S. Patent Nos. 3,984,539; 4,623,541; and 4,816,252.

In one embodiment, plants expressing the polynucleotides and polypeptides described herein are fed to an animal for the treatment and/or prevention of enteric diseases such as colliobacillosis caused by ETEC. By prevention is meant the avoidance of clinical symptoms associated with enteric diseases such as colliobacillosis. By treatment is meant a lessening of the clinical symptoms associated with an enteric disease in an animal receiving the polypeptides, plants, or plant materials of the present invention when compared to an animal having the same enteric disease, but not receiving said polypeptides, plants, or plant materials. Clinical symptoms are not limited to such things as diarrhea and fever, but also include changes in rate of weight gain and/or feed conversion ratios.

In one embodiment, the plants or plant material of the present invention are enterally administered to an animal. For oral administration, the plant or plant part, e.g. leaf, seed or fruit, can be administered without further processing. In other embodiments, the plant or plant material may undergo some processing, for example, to increase palatability and/or digestibility using any of the method known and the art or described herein. For example, the plant material may be dehydrated (e.g. dried), chopped or ground. The plant material can be incorporated into a pellet or a compressed cube. In additional embodiments, the plant or plant material may be mixed with other materials, as for example, a total mixed ration. In another embodiment, the plant or plant materials of the present invention can be incorporated into an artificial milk replacer for administration of a neonate. Methods for the production and feeding of milk replacer are well known in the art and can be found, for example, in US Patent

Nos. 6,348,223; 6,348,222; 5,756,132; 4,961,934; 4,614,653; and 4,269,846. The plants or plant materials can be mixed with liquid to form, for example, a suspension for oral administration such as drenching. In one embodiment, the liquid is milk. In another embodiment, the liquid is water, while in still another embodiment, the liquid is an electrolyte solution, typically an isotonic electrolyte solution such as isotonic saline.

The plants or plant materials of the present invention can also be part of a pre-mix to be added to a feed or food. A pre-mix is a mix of ingredients, typically vitamins, minerals and anti microbials that are then added to a feed or food. In one embodiment, the premix can comprise the plants or plant material of the present invention along with a buffering agent. Likewise the plants or plant materials of the present invention can be used as a food or feed additive. As such, the plants or plant materials can be added to the ingredients of a food or feed as it is being formulated, or can be added to an already existing food of feed such as in top dressing.

In some embodiments, especially those in which the polypeptides of the present invention have been partially or substantially purified, solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the compounds of this invention are ordinarily combined with one or more adjuvants appropriate to the indicated route of administration. If administered *per os*, the compounds can be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets can contain a controlled-release formulation as can be provided in a dispersion of active compound in hydroxypropylmethyl cellulose. In the case of capsules, tablets, and pills, the dosage forms can also comprise buffering agents such as sodium citrate, sodium bicarbonate, or magnesium or calcium carbonate or bicarbonate. Tablets and pills can additionally be prepared with enteric coatings.

Suppositories for rectal administration can be prepared by mixing the materials of the present invention with a suitable non-irritating excipient such as cocoa butter, synthetic mono-, di-, or triglycerides, fatty acids, or polyethylene glycols which are solid at ordinary temperatures but liquid at the rectal temperature, and which will therefore melt in the rectum and release the materials.

In one embodiment, the plants or plant materials of the present invention are administered to neonatal animals in conjunction with a vaccination program for their dams. In this embodiment, the dams are vaccinated at least once, usually at or following midgestation with a vaccine formulated against ETEC. Following birth, the offspring born to the vaccinated dams are administered the compositions of the present invention. Methods for the vaccination of pregnant females against ETEC are known in the art and can be found, for example, in Barman and Sarma, *Indian J. Expt. Biol.*, 37:1132, 1999; Cooper, *Vet. Clin. N. Amer. Food Anim. Prac.*, 16:117, 2000.

It will be recognized by those skilled in the art that the amount of plant material administered will vary with such well known factors as the size of the animal, the severity of the disease, whether the use is for prevention or treatment, the species of animal, the mode of administration, the form of the plant material, for example if it has undergone processing, and whether the plant or plant material is administered in conjunction with additional material to enhance the availability or prevent the degradation of the active ingredient. In general, an animal is administered sufficient plant material to provide at least 5 mg, at least 10 mg, at least 25 mg, at least 50 mg, at least 75 mg or at least 100 mg of anti ETEC antibody on a daily basis. The plants or plant material can be administered in a single dose, in multiple doses, or continuously such as in a feed that is available ad libitum.

Expression cassettes can be designed for stable and optimal expression of antibody molecules in various crops. Expression cassettes can be tailored for specific applications and as such contain various promoter and signal sequences that will determine the antibody molecule processing capabilities, stability of the product and the ease with which it can be recovered. Determination of the proper promoter and signal sequences is routine in the art of molecular biology and can be accomplished by the skilled artisan without undue experimentation.In addition to factors influencing transcription and translation efficiency, recombinant protein accumulation and stability can depend on the compartment of the plant cell chosen for expression. Accumulation in a specific cell compartment can be achieved by attaching to the protein specific targeting signal or by cloning the gene behind tissue-specific promoter. The antibody molecule can be targeted and maintained in any one of the major sites for protein synthesis. In one embodiment, the antibody molecule is targeted to the endoplasmic reticulum (ER) by adding an ER retention signal to the molecule. The signal can be

added to both the light chain and the heavy chains. Alternatively, the signal can be added to the light chain only to favor the association of light chains to heavy chains and restrict secretion to structurally mature assembled antibody molecules. The signal can also be added to the heavy chain only to favor the accumulation of antibody molecules in the ER. In combination with the use of the ER retention signals, the cloning of heavy and light chains under different promoters may allow higher accumulation of antibodies. The ER retention signal "SEKDEL" is particularly preferred.

Table 1

		Promoter								SEKD		Bovine			
	Hybridoma	364	364	364	364	364	364	364	364	364	364	364	364	174	174
		pTMR5	pTMR5	pTMR1	pTMR1	pTMR1	pTMR1	pTMR1	pTMR1	pTMR1	pTMR1	pTMR1	pTMR1	pTMR5	pTMR5
Promot	CMPS			L-	L-	L	L	H	H	L-	L-	L-	L-		
	zmU	L-	L-			H	H	L	L					L-	L-
ER targeting	g-	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Variable	anti-														
	anti-														
	anti-	X	X	X	X	X	X	X	X					X	X
	anti-K88									X	X	X	X		
	anti-														
Constant	synMouse	X	X	X	X	X	X	X	X	X	X				
	synBovine													X	X
												X	X		
ER retention	SEKD	X		X		X		X		L	H	X		X	
	non		X		X		X		X	H	L		X		X

Examples

The following examples are intended to provide illustrations of the application of the present invention. The following examples are not intended to completely define or otherwise limit the scope of the invention.

Example 1

Anti-K99 Antibodies

1.1 Cloning of antibody genes by PCR

Polymerase chain reaction technology and specific oligonucleotide primers were used to clone immunoglobulin genes or regions from hybridoma cDNA. PCR primers specific for both the heavy and light chains of IgG were selected from the Kabat database containing a compilation of mouse heavy chain and light chain sequences available at http://immuno.bme.nwu.edu. Primers for the region encoding the NH2-terminal end of the mature variable region were designed to initiate at the signal sequence and were made with some degeneracy to allow these to be used as "universal primers"(Coloma MJ et al., *J Immunol Methods*, 152:89, 1992). The 3' primers used for the specific PCR amplification of the variable regions were designed from conserved sequences of the first constant domain (CH1) of both the light and heavy chains (Dattamajumdar et al., *Immunogenetics*, 43:141, 1996). Table 2 shows the sequence and hybridization location of the primers used for the PCR cloning of the mouse immunoglobulin light and heavy variable regions.

Total RNA was isolated from 10^7 hybridoma cells 2BD4E4 (ATCC HB-8178; K99 F5 pili) using a procedure based on the RNeasy mini kit (Qiagen, Hilden Germany). Cells were lysed by passing them through an 18-gauge needle at least 5 times. Poly-A+ RNA was purified from RNA using the Oligotex mRNA mini kit (Qiagen). Approximately 20 ng was used to generate the first strand cDNA using the Clontech cDNA synthesis kit (Clontech Laboratories, Inc., Palo Alto, CA). mRNA was preincubated with oligo (dT_{30}) at 70°C for 2 minutes and then cooled on ice for 2 minutes. The buffer (50 mM, Tris-HCl, pH 8.3; 75 mM, KCl; 6 mM, MgCl2; 2 mM DTT; 1mM dNTPs, final concentration) and Moloney Murine Leukemia Virus reverse transcriptase (200 units, Superscript, BRL SuperScript II Rnase H, Rockville, MD, USA) were added in a 10 µl reaction volume and the solution was incubated at

42°C for one hour. After the reaction, 10 µl TE buffer (10mM Tris-HCl, 1mM EDTA) was added.

Next, one 20th (or 1 µl) of the first-strand cDNA reaction was added to a 20 µL PCR reaction mix containing immunoglobulin specific primers. Primers used are listed in Table 2. The mixture was amplified using Taq polymerase (0.025 U), 0.25 µM of each primer and 0.2 µM of each dNTP for 28 cycles. The temperature and times used for PCR were as follows: denaturation at 94°C for 15 seconds; annealing at 50°C for 15 seconds; extension at 68°C for 1 min. Primers corresponded to universal heavy and light chain primers specific for mouse immunoglobulin signal sequences and constant regions for sense and antisense primers respectively.

Separate tubes were used for light chain and heavy chain amplifications. An aliquot of the PCR products was purified (Qiagen), sequenced and then primers were designed for the sense primer (based on the FR1 region) and antisense primer (based on the 5'-end of the constant region) for both chains. PCR products were cloned into pTOPO (Invitrogen, Carlsbad, CA). After confirmation of the sequence using the M13 forward and reverse primers (New England Biolabs, Beverly, MA), variable regions were digested with SpeI/XhoI for light chain and SpeI/ AccI for the heavy chain and the resulting fragments were cloned into the desired expression vectors.

Table 8 contains a listing of the sequence ID numbers for the variable region sequences. The sequences are complete variable regions starting at the first codon of the first framework region and ending with the last codon of the fourth framework region of the variable region.

Table 2 Primers for PCR amplification of antibody mRNA from hybridomas

Primer	Region	ID	SEQUENCE	SEQ ID #
Forward	VL	MLALT2	accatggattttcaagtgcagattttcag	19
		MLALT3	caccatggagwcacakwctcagtgtctttrt	20
		MLALT4	caccatgkccccwrctcagytyctkgt	21
		MLALT5	caccatgaagttgcctgttaggctgttg	22
	VH	MH1	atatccaccatggratgsagctgkgtmatsctctt	23
		MH2	atatccaccatgracttcgggytgagctkggtttt	24
Reverse	VL	33615	gaagatctagacttactatgcagcatcagc	25
	VH	MVG1R	ggcagcactagtaggggccagtggata	26
		MVG2R	gaggarccactagtatctccacacmcaggggccag	27

VL = variable light chain
VH = variable heavy chain

1.2 Cloning of antibody genes into expression vectors

All plasmid manipulations were carried out according to standard procedures (Sambrook et al., *Molecular Cloning*, 2nd ed, Cold Spring Harbor Laboratory Press, 1989). All vectors for plant expression contained the 19 amino acid endoplasmic reticulum transit peptide (SS) [MRVLLVALALLALAASATS] (SEQ ID NO. 28) from the maize γZein gene behind a plant promoter as well as the maize phosphoenolpyruvate carboxylase intron #9 (Hudspeth and Grula, *Plant Mol. Biol.*, 12:579-592, 1989) immediately before the CaMV 35S terminator sequence. Within the 5' untranslated leader region all constructs contained a plant consensus translational sequence ACC ATG AG.

1.3 Agrobacterium binary vectors

For Agrobacterium inoculation, the heavy and the light chain cassettes were cloned in between the T-DNA borders of the binary vector pNOV2117. pNOV2117 contains a plant-expressible *pmi* gene driven by the maize ubiquitin promoter for selection on mannose (Negrotto et al., *Plant Cell Rpts.*, 19:798-803, 2000).

All binary expression clones were constructed in a three-way ligation with the 9,172 bp *Hind*III/*Acc*65I fragment from pNOV2117. The plasmid pTMR108 contained both the heavy and light chains with the endoplasmic reticulum retention signal under the control of the γZein promoter. pTMR108 was made by ligating the 1613 bp *Hind*III/*Mlu*I fragment from pTMR100 and the 2281bp *Mlu*I/*Acc*65I fragment from pTMR142 with the pNOV2117 vector described above. The plasmid pTMR110 only differed from pTMR108 by the absence of the endoplasmic reticulum retention signal. pTMR110 resulted from the ligation of the 1597 bp *Hind*III/*Mlu*I fragment of pTMR104 and the 2263bp *Mlu*I/*Acc*65I fragment from pTMR144 with the pNOV2117 vector. The clone pTMR109 contained both the heavy and light chain with the endoplasmic reticulum retention signal under the control of the Ubiquitin promoter. pTMR109 was made by ligating the 2958 bp *Hind*III/*Mlu*I fragment from pTMR101 and the 3608 bp *Mlu*I/*Acc*65I fragment from pTMR143 with the pNOV2117 vector. The clone pTMR111 only differed from pTMR109 by the absence of the endoplasmic

reticulum retention signal. pTMR111 resulted from the ligation of the 2940 bp *Hind*III/*Mlu*I fragment from pTMR105 and the 3590 bp *Mlu*I/*Acc*65I fragment from pTMR145 with the pNOV2117 vector.

1.4 Cloning of plant promoters and terminator sequences for plant expression cassettes

The starting plasmid for PCR amplification was pNOV4097 containing the γZein promoter, the γZein transit peptide, the Ubiquitin promoter and the terminator (PEPCTerm). The γZein promoter together with the γZein transit peptide sequence was PCR-amplified to introduce *Mlu*I and *Spe*I restriction sites at the 5'and 3'ends respectively with the following primers: 5'-acgcgtcgatcatccaggtgcaac-3' (SEQ ID NO. 29), and 5'-actagtggcgctcgcagcgaga-3' (SEQ ID NO. 30). The 746bp PCR product was cloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) giving rise to pTA gZpromSS.

Similarly, the PEPCintron 35Sterm was PCR-amplified to introduce *Age*I and *Mlu*I restriction sites at the 5'and 3' ends respectively with the following primers: 5'-accggttctgttctgcacaaagtgt-3' (SEQ ID NO. 31) and 5'-acgcgtttgtacccctggatt-3' (SEQ ID NO. 32). The 206 bp PCR product was cloned into pCR2.1 TOPO vector giving rise to pTA pepc35Sterm.

A partial fragment of the Ubiquitin promoter was PCR-amplified to introduce a 5' *Mlu*I restriction site. The internal *Xho*I restriction site was preserved. A fragment of 741bp was amplified with the following primers: 5'-acgcgtttgcatgcctgcagtg-3' (SEQ ID NO. 33) and 5'-agtccaacggtggagcggaact-3' (SEQ ID NO. 34). The PCR product was cloned into pCR2.1 TOPO vector giving rise to pTA MluI zmUbi.

1.5 Cloning of the light chain expression cassette

The sequence encoding the variable and the constant regions for the light chain of the anti-K99 antibody was synthesized chemically with optimized codon usage for expression in maize. A *Spe*I restiction site was introduced at the 5' end of the variable region and a *Xho*I restriction site was introduced at the 3' end of the variable domain in the highly conserved KLEIK (SEQ ID NO. 56) motif of FR4. An *Age*I restriction site was added at the 3'end of the constant region. The endoplasmic reticulum retention signal (SEKDEL, SEQ ID NO. 35) was added to the 3' end of the constant region. This clone was named pK99LC.

The following cloning strategy was developed: the plant promoter would be brought as a *Hind*III/*Bam*HI fragment, the γZein transit peptide sequence as a *Bam*HI/*Spe*I fragment, the variable region as a *Spe*I/*Xho*I fragment, the terminator as an *Age*I/*Mlu*I fragment and the endoplasmic reticulum retention signal could be removed, if desired, by oligonucletide replacement as a *Pci*I/*Age*I fragment.

In a first step, the multiple cloning site of pUC19 was changed into *Hind*III-*Bam*HI-*Spe*I-*Age*I-*Mlu*I-*Eco*RI in order to facilitate the cloning of the light chain. The following annealed oligonucleotides with *Hind*III/*Eco*RI staggered ends were cloned into the *Hind*III/*Eco*RI sites of the pUC19 vector to give rise to the clone pKappa vector:

5'-agcttggatccactagtaccggtacgcgtg-3' (SEQ ID NO. 36) and

5'-aattcacgcgtaccggtactagtggatcca-3' (SEQ ID NO. 37).

A terminator was added to pK99LC to build pK99LC-term in a three way ligation as follows: *Spe*I/*Age*I pK99LC fragment, *Spe*I/*Mlu*I pKappa vector and *Age*I/*Mlu*I pTA pepc35Sterm. The endoplasmic reticulum retention signal was removed from the pK99LC-term vector by ligating the 597bp *Hind*III/*Pci*I fragment from pK99LC-term, the 72bp *Pci*I/*Age*I annealed oligonucleotides and the 2841bp *Age*I/*Hind*III fragment from pK99LC-term. The resulting plasmid was named pK99LCw/oSEKDEL-term. The following oligonucleotides were annealed to make the 72bp *Pci*I/*Age*I fragment:

5'-catgtgaggccacccacaagacctccacctccccaatcgtgaagagcttcaaccgcaacgagtgctgataga-3' (SEQ ID NO. 38) and

5'-ccggtctatcagcactcgttgcggttgaagctcttcacgattggggaggtggaggtcttgtgggtggcctca-3' (SEQ ID NO. 39).

1.6 Cloning of the heavy chain expression cassette

The sequence encoding the heavy chain variable region, from the anti-K99 antibody, and the heavy chain constant region, which was derived from a consensus sequence of mouse IgG1 (Kabat et al., U.S. Dept Health and Human Services, U.S. Government Printing Offices 1987) was synthesized chemically with optimized codon usage for expression in maize. A *Spe*I restriction site was introduced at the 5' end of the variable region and an *Acc*I restriction site was introduced in the CH1 domain

corresponding to amino acid 9. This sequence did not include an endoplasmic reticulum retention signal. This clone was named pK99HC.

The following cloning strategy was developed: The plant promoter was cloned as a *Mlu*I/*Bam*HI fragment, the γZein transit peptide sequence as a *Bam*HI/*Spe*I fragment, the variable domain as an *Spe*I/*Acc*I fragment, the terminator as a *Sac*I/*Acc*65I fragment and the endoplasmic reticulum signal sequence added as a *Xma*I/*Sac*I oligonucleotide.

In a first step, the multiple cloning site of pUC19 was changed into *Hind*III-*Mlu*I-*Bam*HI-*Spe*I-*Sac*I-*Acc*65I-*Eco*RI in order to facilitate the cloning of the heavy chain. The following annealed oligonucleotides with *Hind*III/*Eco*RI staggered ends were cloned into the *Hind*III/*Eco*RI sites of the pUC19 vector to give rise to the clone pIgG1vector using the following annealed oligonucleotides: 5'-agcttacgcgtggatccactagtgagctcggtaccg-3' (SEQ ID NO. 40) and 5'-aattcggtaccgagctcactagtggatccacgcgta-3' (SEQ ID NO. 41).

The endoplasmic reticulum retention signal was then introduced at the 3'end of the constant region by a three way ligation with the *Spe*I/*Xma*I fragment from pK99HC, the *Xma*I/*Hind*III annealed oligonucleotides and the *Spe*I/*Hind*III pIgG1 vector. This clone was named pK99HC with SEKDEL (SEQ ID NO. 35). The following primers were annealed to form the 5' *Xma*I-3' *Hind*III fragment harboring an internal *Sac*I restriction site at the 3'-end:
5'-ccgggcaagtccgagaaggacgagctgtgataggagctcaaggtaccgaattca-3' (SEQ ID NO. 42) and 5'-agcttgaattcggtaccttgagctcctatcacagctcgtccttctcggacttgc-3' (SEQ ID NO. 43). The terminator was added to pK99HC in a three way ligation as followed: *Spe*I/*Sac*I pK99HC, *Sac*I/*Acc*65I pNOV3402 (term) + *Spe*I/*Acc*65I pIgG1 vector. This clone was named pK99HC-term.

1.7 Light Chain Plant Expression Vector

The plasmid pTMR100 contained the light chain with the endoplasmic retention signal under the control of the γZein promoter. The 777 bp *Hind*III/*Spe*I fragment from the clone pTA-γZeinSS, encompassing the γZein promoter and γZein signal sequence, was cloned into the corresponding sites of the 3516 bp *Hind*III/*Spe*I fragment from pK99LC:term. The clone pTMR104 differed from pTMR100 by the absence of the endoplasmic reticulum retention signal. pTMR104 resulted from the ligation of the 777

bp *Hind*III/*Spe*I fragment from pTA-γZeinSS into the corresponding sites of the 3498 bp fragment from pK99LCw/oSEKDEL:term.

The plasmid pTMR101 contained the light chain with the endoplasmic retention signal under the control of the Ubiquitin promoter. The 2004 bp *Hind*III/*Bam*HI fragment of pNOV2117, encompassing the maize ubiquitin promoter,was ligated with the 3576 bp *Hind*III/*Bam*HI fragment from the clone pTMR100. The plasmid pTMR105 differed from pTMR101 by the absence of the endoplasmic reticulum retention signal. pTMR105 resulted from the ligation of the 4706 bp *Spe*I/*Mlu*I fragment from clone pTMR101 with the 857 bp *Spe*I/*Mlu*I fragment from pK99HCw/oSEKDEL:term.

1.8 Heavy chain plant expression vectors

The plasmids pTMR142 and pTMR144 contained the variable region of anti-K99 and the heavy constant region under the control of the maize γZein promoter whereas the clones pTMR143 and pTMR145 contained the variable region of anti-K99 and the constant region under the control of the Ubiquitin promoter. Additionally, both pTMR143 and pTMR145 included the endoplasmic reticulum retention signal.

The plasmid pTMR144 was made by ligating a 740 bp *Mlu*I/*Spe*I fragment from the plasmid pTA-γZeinSS, encompassing the γZein promoter and γZein signal sequence into the corresponding sites of the 4174 bp pK99HC-term vector. The plasmid pTMR142 differed from pTMR144 only in the 3'-end of the constant region that contained the additional endoplasmic reticulum retention signal. This clone was made by ligating a 3581 bp *Spe*I/*Sac*I fragment from pTMR144 and a 1348 bp *Spe*I/*Sac*I fragment from pK99HC.

The plasmid pTMR145 was made by ligating the 4230 bp *Mlu*I/*Bam*HI fragment from pTMR144, the 709bp *Mlu*I/*Xho*I fragment from pTA-MluI zmUbi encompassing the 5'region of the ubiquitin promoter and the 1298 bp *Xho*I/*Bam*HI fragment from pNOV2115 containing the 3'region of the promoter. The clone pTMR143 differed from pTMR145 only in the 3'-end of the constant region that contained the additional endoplasmic reticulum retention signal. The clone was built by assembling the 4908 bp *Spe*I/*Sac*I fragment of pTMR145 into the corresponding sites of the 1348 bp *Spe*I/*Sac*I

fragment from pK99HC with SEKDEL. A summary of the various vectors is given in Table 3.

Table 3 Expression Constructs

Name	Construct	Vector
pTMR100	γZein promoter:K99Kappa + SEKDEL:term	pUC
pTMR101	zmUbi promoter: K99Kappa + SEKDEL:term	pUC
pTMR104	γZein promoter:K99Kappa:term	pUC
pTMR105	zmUbi promoter: K99Kappa:term	pUC
pTMR142	γZein promoter: K99Heavy + SEKDEL:term	pUC
pTMR143	zmUbi promoter:K99Heavy + SEKDEL:term	pUC
pTMR144	γZein promoter : K99Heavy:term	pUC
pTMR145	zmUbi promoter: K99Heavy:term	pUC
pTMR110	pTMR104 + pTMR 106 in pNOV2117	Agrobacterium vector
pTMR146	pTMR100 + pTMR 142 in pNOV2117	Agrobacterium vector
pTMR147	pTMR101 + pTMR 143 in pNOV2117	Agrobacterium vector
pTMR148	pTMR104 + pTMR 144 in pNOV2117	Agrobacterium vector
pTMR149	pTMR105 + pTMR145 in pNOV2117	Agrobacterium vector
pTMR156	pTMR101 + pTMR145 in pNOV2117	Agrobacterium vector
pTMR160	pTMR105 + pTMR143 in pNOV2117	Agrobacterium vector

1.9 Transformation and Expression in Plants

Two independent antibody chains or antibody domains were expressed and assembled in plants using normal cellular processing. Antibody proteins were either targeted to the plant apoplast, or, in the case of constructs that have an ER retention signal, targeted to organelles within the plant cell. Vacuolar targeting signals in the form of carboxyl-terminal propeptides have been described previously (Muntz, *Plant Mol. Biol.*, 38:77-99, 1998; Neuhaus et al., *Plant Mol. Biol.*, 38:127-144, 1998).

1.10 *Agrobacterium*-mediated transformation of maize

Transformation of immature maize embryos was performed essentially as described in Negrotto et al., *Plant Cell Reports* 19:798, 2000. For this example, all media constituents are as described in Negrotto et al., *supra*. However, various media constituents described in the literature may be substituted.

1.10.1 Transformation plasmids and selectable marker:

The genes used for transformation were cloned into a vector suitable for maize transformation. Vectors used in this example contained the phosphomannose isomerase

(PMI) gene for selection of transgenic lines (Negrotto et al. *supra*). A representation of the vector used for *Agrobacterium* transformation can be found in Figure 2

1.10.2 Preparation of Agrobacterium tumefaciens:

Agrobacterium strain LBA4404 (pSB1) containing the plant transformation plasmid was grown on YEP (yeast extract (5 g/L), peptone (10g/L), NaCl (5g/L), 15g/l agar, pH 6.8) solid medium for 2-4 days at 28°C. Approximately 0.8X 10^9 *Agrobacterium* were suspended in LS-inf media supplemented with 100 µM As (Negrotto et al., *supra*). Bacteria were pre-induced in this medium for 30-60 minutes.

1.10.3 Inoculation

Immature embryos from A188 or other suitable genotype were excised from 8-12 day old ears into liquid LS-inf + 100 µM As. Embryos were rinsed once with fresh infection medium. *Agrobacterium* solution was then added and embryos were vortexed for 30 seconds and allowed to settle with the bacteria for 5 minutes. The embryos were then transferred scutellum side up to LS- inf + 100 µM As medium and cultured in the dark for two to three days. Subsequently, between 20 and 25 embryos per petri plate were transferred to LSDc medium (Negrotto et al., *supra*) supplemented with cefotaxime (250 mg/l) and silver nitrate (1.6 mg/l) and cultured in the dark for 28°C for 10 days.

1.10.4 Selection of transformed cells and regeneration of transformed plants

Immature embryos, producing embryogenic callus were transferred to LSD1M0.5S medium (Negrotto et al., *supra*). The cultures were selected on this medium for 6 weeks with a subculture step at 3 weeks. Surviving calli were transferred to Reg1 medium supplemented with mannose (Negrotto et al., *supra*). Following culturing in the light (16 hour light/ 8 hour dark regiment), green tissues were then transferred to Reg2 medium (Negrotto et al., *supra*) without growth regulators and incubated for 1-2 weeks. Plantlets were transferred to Magenta GA-7 boxes (Magenta Corp, Chicago IL.) containing Reg3 medium (Negrotto et al., *supra*) and grown in the light. After 2-3 weeks, plants were tested for the presence of the PMI genes and other genes of interest by PCR. Positive plants from the PCR assay were transferred to the greenhouse.

1.11 Rice transformation

Oryza sativa L. japonica cv. Taipei 309 was used for the production of transgenic plants. Callus induction, cell suspension initiation and maintenance followed protocols previously described by Zhang, *Plant Cell Rpt.*, 15:68, 1995. Calli or suspension cells were subjected to particle bombardment 3-5 days after the previous subculture or to *Agrobacterium*-mediated transformation. Calli or suspension cell clusters, between 1 and 3 mm in diameter, were placed on NBO osmotic medium for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto modified NB selection medium containing 25 g/l of mannose and 5 g/l of sucrose. Following an additional 30-40 days, newly formed callus was then transferred to PR pre-regeneration medium for 7 days in the dark. Growing callus was then subcultured onto RN regeneration medium for a period of 14-21 days under a 16-h photoperiod. The composition of the media has been described previously (Chen et al, *Plant Cell Rpt.*, 18:25, 1998).

Tissues were bombarded with gold microprojectiles onto which was precipitated a mixture of plasmids. The phosphomannose isomerase gene under the control of the Zea mays ubiquitin promoter was used as selectable marker. Co-transformation experiments were carried out with the selectable marker plasmid DNA together with pTMR101 and pTMR143 or with pTMR105 and pTMR145 plasmid DNAs at the molar ratio of 1:6:6 as described by Chen et al., *Nature Biotech.*, 16:1060, 1998. One µg of total mixed DNA was used for a target plate. Gene coating and transfer was achieved using the Biolistic PDS-1000 system (Bio-Rad. Hercules, CA) according to Zhang et al., *Molec. Breeding*, 4:551, 1998 and Chen et al., *Plant Cell Rpt.*, 18:25, 1998. A total of 50 transformed callus were obtained for each construct set.

1.12 Protein extraction from rice callus and expression analysis

Rice cells were frozen in liquid nitrogen, ground to fine powder in 1.5 ml microfuge tubes with a Teflon hand grinder, vortexed and placed on ice with extraction buffer (EB: 10mM Tris 7.5, 100mM NaCl, 5mM EDTA) in the presence of plant protease inhibitor cocktail mix (Sigma, St. Louis, MO, Cat# P9599). 1.8µl of EB buffer was used per mg of rice callus and 2 µl of protease inhibitor per 100ul of EB buffer.

The Bradford method (Bio-Rad, Hercules, CA) was used according to the manufacturer's instructions for protein determination. Equal amounts of protein (40 µg/lane), along with Mr standard (BenchMark, Invitrogen, Carlsbad, CA) were subjected to SDS-PAGE using precast 4-12% (w/v) gels (NuPage, Invitrogen). The mouse IgG1 (Lampire Biological Laboratories, Pipersville, PA) was used as a standard. Gels were electroblotted onto nitrocellulose membranes (Invitrogen) and stained with Ponceau S to visualize the transfer. The blots were incubated in a blocking solution consisting of 10% (w/v) nonfat dry milk in TTBS (20mM Tris 7.5; 100mM NaCl; 0.1% TWEEN-20) for 1h at RT or overnight at 4°C. After rinsing with TTBS buffer, rabbit anti-mouse antibody (Zymed Laboratories, South San Francisco, CA, Cat# 61-6500) was added at a concentration of 1µg/ml and incubated for 1 h. After three, 10-min washes in TTBS, the membrane was incubated with 0.2 µg/ml alkaline phosphatase-conjugated goat anti-rabbit (Zymed Laboratories, Cat# 656122). The blot was further washed as indicated above, and the immunolabeled proteins were detected using the alkaline substrate (Promega, Madison, WI).

Separation in denaturing conditions showed that the subunits of the plant-derived anti K99 antibody were identical in size to those present in the supernatant of the hybridoma. Results obtained under reducing conditions (Figure 3) show the accumulation of bands of various sizes corresponding to the assembly intermediates between light and heavy chains.

1.13 Antigen Binding Assay

An Elisa assay was performed to test the binding capacity of the assembled antibody to the K99 antigen (see Example 2.13). The results are given in Table 4a and show that extracts from plants expressing the heavy and light chains reacted with the antigen.

Table 4a Antigen-binding Elisa assays on transgenic rice callus. Samples were diluted 1:2 in 2% milk in PBS buffer. Plates were sensitized with K-99 antigen and milk was used as blocking reagent.

Extract	Optical Density
Anti-K99 from hybridoma cells	1.800
Transgenic callus 1	0.214
Transgenic callus 2	0.122
Transgenic callus 3	0.208

Extract	Optical Density
Negative control	0.050
Non-transgenic callus	0.050

1.14 Rice Callus Expression

Rice callus were transformed by *Agrobacterium* containing four different constructs: pTMR147, pTMR149, pTMR156 and pTMR160. Transgenic callus were recovered and analyzed for antibody expression by enzyme-linked immunoassay (ELISA) and by immunoblotting in several independent transgenic lines. ELISA analyses showed that transgenic rice callus expressed functional antibody and that the expression ranged between approximately 200 to 1200 ng/mg TSP (Table 4b).

Typical results from Western blots of rice callus are shown in Figure 26. No bands appeared on Western blots for non-transgenic rice callus under non-reducing and reducing conditions. Results under reducing conditions are shown in figure 26A. Two major bands were present at about 50 and 25 kDa and exhibited similar size than the ones from the corresponding antibody purified from hybridoma cells. The upper band was identified as the heavy chain; the lower band was identified as the light chain.

Table 4b Expression levels of anti-K99 determined by ELISA in rice callus transformed with 4 different constructs. Microplates were coated with a rabbit anti-mouse IgG-kappa (L+Fc) or with a rabbit anti-mouse IgG1 (HL+ Fc). In all cases, the binding was revealed with a rabbit anti-mouse IgG- alkaline phosphatase-conjugated (Fc specific). 2 µl of callus extract was loaded per well for the quantitative ELISA. TSP: total soluble protein.

Plasmids		HL+Fc ng/mg TSP	L+Fc ng/mg TSP
pTMR147	Callus 1	770	770
	Callus 2	667	555
	Callus 3	1442	1203
	Callus 4	667	555
pTMR149	Callus 5	625	625
	Callus 6	357	286
	Callus 7	896	746
	Callus 8	1083	1000
pTMR156	Callus 9	707	543
	Callus 10	600	500
	Callus 11	1207	862
	Callus 12	1027	959
pTMR160	Callus 13	823	760
	Callus 14	1053	790
	Callus 15	1094	938

Plasmids		HL+Fc ng/mg TSP	L+Fc ng/mg TSP
Control	Callus 16	0.09	0.07
PBS buffer		0.09	0.04

Example 2

K88 Antibody

2.1 Characterization of hybridoma lines

All hybridoma cell lines containing antibodies against K88 were obtained from Rural Technologies (University of South Dakota, Vermillion, SD). Hybridomas 17/44, 7/46, and 36/41 have specificity against K88 (F4) C domain, K88 (F4) Va domain, and K88 (F4) Va domain respectively (Sun, et al., *Infection and Immunity,* 68:3509-3515, 2000).

2.1.1 Hybridoma culture

Hybridoma cell lines were grown in RPMI-1640 medium supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin (10,000U/ml) and 15% fetal bovine serum (FBS) all obtained from Invitrogen, Carlsbad, CA. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For subcloning by limiting dilution, the concentration of viable cells was determined by trypan blue exclusion and cells were diluted in culture media to a concentration of 0.5-1.0x10^6cells/ml. From this, two further 10-fold dilutions were made and each dilution was distributed to two 96 well plates (100 µl/well) containing a macrophage feeder layer. Feeder layers were prepared a day in advance by performing a peritoneal lavage of two BALB/c mice and adding 100 µl of the collected fluid to each well of six 96 well plates. Supernatants were collected about 10 days later from wells containing only one colony per well, and tested for reactivity with the appropriate antigen.

2.1.2 ELISA

Antibodies produced from hybridomas were characterized using an ELISA. ELISA assays were performed in 96 well microtiter plates (Immunol IV; Dynex, Franklin, MA). Wells were coated with antigen diluted 1:50 in carbonate coating buffer (0.15 M sodium carbonate, 0.35 M sodium bicarbonate, 0.03 M sodium azide, pH 9.6) and incubated overnight at 4°C temperature. Coated plates were washed and blocked

with 1% polyvinyl alcohol (PVA) for 1 hr at 37°C prior to the addition of the monoclonal antibody. All reagents used in this assay were diluted in PBS + 0.1%Tween 20 (PBST) and after each incubation step wells were washed with this buffer. After blocking and washing, antibody was added at a 1:1000 dilution and allowed to incubate for 1 h at 37°C. Excess antibody was removed by three washing steps and the wells were blocked prior to the addition of the secondary antibody. Horseradish peroxidase (HRP)-labeled goat-anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at a 1:10,000 dilution was added for 1 hr for 37°C . After washing the enzyme substrate 3,3',5,5'-Tetramethyl Benzidine (TMB) was added. The reaction was stopped by the addition of 1 M sulfuric acid and the optical density was read at a wavelength of 450nm using a BioRad Microplate reader, Model 3550.

Alternatively, a 96 well plate was loaded with 50 µl of *E. coli* carrying plasmids 987P, K99 or F41. Bacteria were grown in Minimal Medium to a concentration of 0.7 $O.D._{600}$. The plate was incubated overnight at 4°C. Next day, plates were washed three times with PBS. Plates were then blocked using 300 µl of 10% skim milk in PBS for 1 hour at 37°C. Plates were washed three times with PBS/Tween 20 (0.05%) and three times with PBS. Fifty µl of 2% skim milk in PBS was added to the plates. Fifty µl of supernatants from each hybridoma was added to each well of the ELISA plate. The antigen-antibody reaction was incubated for 1 hour at RT, in a shaker. Plates were washed three times with PBS/Tween 20 (0.05%) and three times with PBS. Fifty µl of a 1:1000 dilution of an anti-mouse IgG-HRPO conjugated diluted in 2% skim milk in PBS was added to each well and incubated for 1 hour at RT in a shaker. Plates were washed three times with PBS/Tween 20 (0.05%) and three times with PBS. Fifty µl of ABTS was added to each well. The reaction was stopped when color was developed using 50 µl of 1% SDS. The OD was read at 405 nm.

2.1.3 Western blots

The purity of extracted pili (fimbriae) was determined on a 12% polyacrylamide gel (Gibco, Invitrogen, Carlsbad, CA). Briefly, antigen preparations were boiled in SDS sample buffer and loaded into the stacking gel. Protein bands were resolved by electrophoresis performed for 1.5 hr at 100V. Bands were visualized on the gels using Coomassie Blue stain. For Western blots, the proteins in the polyacrylamide gels were

transferred to PVDF membranes using a wet transfer method (BioRad, Hercules, CA). The transfer was carried out at 4°C at 60V for 2 hrs. After transfer was completed, the nitrocellulose membranes were washed in PBS and blocked in 1% PVA overnight at 4°C. After blocking, membranes were incubated with the antibody for 1 hr at room temperature. Dilutions of antibody were prepared in PBST. Membranes were then blocked and washed again before the addition of the detecting antibody, HRP conjugated goat anti-mouse at a 1:10,000 dilution, for 1 hr at room temperature. Antibody-antigen reactions in the membranes were visualized using ECL Western Blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) on x-ray film.

2.1.4 Results

Hybridoma 17/44 has been reported to bind the conserved domain of pili from K88 and 7/46 and 36/41 have been reported to bind the variable domain "c". These hybridoma cell lines were expanded and the reactivity of supernatants against K88 was determined in ELISA and Western Blots. Results from these assays showed that supernatants from hybridomas 17/44 and 7/46 were reacting with K88. These cell lines were subcloned in preparation for mRNA extraction and subsequent PCR cloning of genes coding for VH and VL regions. Supernatant fluids from hybridoma cell line 36/41 showed the best positive reactivity in ELISA assays and were selected for further subcloning. Approximately 20-30 subclones for each hybridoma cell line were screened for reactivity to K88 using ELISA. Of these, 3 clones from each cell line were chosen based on high OD and viability/growth properties. Amplification of these subclones was performed as follows: cells from each well were taken from the 96-well plate and transferred to a well in a 24-well plate. Subclones were grown for 3 to 6 days in RPMI-1640 + 15% FCS supplemented with 1% L-glutamine, and 1% Pen/Strep. A new ELISA was performed and all subclones were confirmed positive. Subclones K88-7/46: 3 and 9; K88-17/44: 6 and 33; K88-36/41: 1 and 23 were further expanded using a 25 cc flask followed by a 75 cc flask. When the growth of the cells was adequate, hybridomas were collected and resuspended in culture media containing 8% DMSO and stored frozen in liquid nitrogen.

2.2 Cloning of antibody genes by PCR

The procedures used were that same as in Example 1.1 with the following changes. The hybridoma cells lines used were 36/41, 7/46, 17/44. For the cloning of K88 from hybridoma 36/41, the following primers were used: MLALT2 (SEQ ID NO. 19) and 33615 (SEQ ID NO. 25) for the light chain, and MVG1R (SEQ ID NO. 26) and MH1 (SEQ ID NO. 23) for the heavy chain. For the cloning of the K88 antibody from hybridoma 7/46, the following primers were used: MLALT2 (SEQ ID NO. 19) and 33615 (SEQ ID NO. 25) for the light chain and MH2 (SEQ ID NO. 24) and MVG1R (SEQ ID NO. 26) or MVG2R (SEQ ID NO. 27) for the heavy chain. For the cloning of the K88 antibody from hybridoma 17/44, the primers MH2 (SEQ ID NO. 24) and MVG1R (SEQ ID NO. 26) were used to clone the heavy chain. The light chain was cloned by using the 5' RACE kit (Clonetech) using the following primers: 33615 (SEQ ID NO. 25) and the 5' PCR Primer IIA (5'-aagcagtggtatcaacgcagagt-3', SEQ ID NO. 44) that anneals with the SMART IIA primer (5'-aagcagtggtatcaacgcagagtacg cggg-3', SEQ ID NO. 45). This latter primer was ligated to the 5' end of the cDNA during the cDNA synthesis reaction. The following parameters were used for the 5'RACE reaction: The first strand synthesis reaction was carried out using 3 µl of total RNA from hybridoma cells 17/44 and 1µl of 33615 primer (SEQ ID NO. 25) (10 µM), 1 µl of Smart IIA primer (SEQ ID NO. 45) (10 µM) to which was added 2 µl 5x 1st Strand Buffer, 1 µl DTT (20 mM), 1 µl dNTP (10 µM), 1 µl of SuperscriptII (200 u/µl), and 10 µl of TE buffer (10 mM TrisCl, 1mM EDTA, pH 7.4). After an incubation of 2 min at 72°C and a brief incubation on ice, the reaction was carried out at 42°C for 1hr. For PCR, the reaction mixture contained 1 µl of first strand DNA, 5 µl of 10X Taq buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl dNTPs (10µM), 1 µl of 5' PCR Primer IIA (SEQ ID NO. 44) (10µM), 1 µl of primer 33615 (SEQ ID NO. 25) (10µM), 37.5 µl of dH2O, and 1 µl of Taq polymerase (5U/µl). The PCR reaction mixture was held at 94°C for 5 min followed by 28 cycles of 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1min.

2.3 Cloning of K88 antibody genes.

Three antibodies against K88, antibody 7/46, 17/44 and 36/41, were cloned. Variable regions of the heavy chains were cloned with the following reverse primer (K99HC-3' aagtagacagatgggggtgtcg SEQ ID NO. 46). The forward primers varied for

each antibody and were as follows: Antibody 7/46: K88_746_VAR_H5' gccactagtgaagtgaagcttgaggag (SEQ ID NO. 47); Antibody 17/44: K88_1744_VAR_H5' gccactagtgatgtgcagctggtgga (SEQ ID NO. 48); Antibody 36/41: K88_3641_VAR_H5' gccactagtgaggtccagctgcagcag (SEQ ID NO. 49).

The amplification of the variable region of the light chain was performed with the following sets of primers: Antibody 7/46: K88_746_VAR_L5 ccactagtgaaattgtgctcacccag (SEQ ID NO. 50) and K88_746_VAR_L3 ttatctcgagctttgtccccgagccgaa (SEQ ID NO. 51). For antibody 36/41: K88_3641_VAR_L5 gccactagtgaaaatgtgctcacccag (SEQ ID NO. 52) and K88_3641_VAR_L3 ttatctcgagcttggtgcctccaccgaa (SEQ ID NO. 53). For antibody 17/44: K88_1744_VAR_L5 gccactagtgacattgtgatgtcacag (SEQ ID NO. 54) and K88_1744_VAR_L3 ttatctcgagcttggtcccagcaccgaacg (SEQ ID NO. 55).

The cloning of the light chain and the heavy chain variable regions from the antibody 36/41 into the vector pCR2.1-TOPO resulted into pTMR524 and pTMR525 respectively. The cloning of the light chain and the heavy chain variable regions from the antibody 7/46 into the vector pCR2.1-TOPO resulted into pTMR526 and pTMR527 respectively. The cloning of the light chain and the heavy chain variable regions from the antibody 17/44 into the vector pCR2.1-TOPO resulted into pTMR528 and pTMR529 respectively.

2.4 Cloning of antibody genes into expression vectors

The methods used were the same as described in Example 1.2

2.5 Agrobacterium binary vectors

For Agrobacterium inoculation, the heavy and the light chain cassettes were cloned in between the T-DNA borders of the binary vector pNOV2117. pNOV2117 contains a plant-expressible *pmi* gene driven by the maize ubiquitin promoter for selection on mannose (Negrotto et al., *Plant Cell Reports*, 19:798-803, 2000). Binary expression clones containing the Ubiquitin and/or γZein promoters were constructed in a three-way ligation with the 9,172 bp *Hind*III/*Acc65*I fragment from pNOV2117 as reported in Table 5. Constructs containing the CMPS promoter and CMPS/Ubiquitin promoter combinations were cloned in either a three-way or a four-way ligation with the 9,172 bp HindIII/Acc65I fragment from pNOV2117 as detailed in table 5.

2.6 Cloning of Plant promoters and terminator sequences for plant expression cassettes.

All components were cloned as described in section 1.4. The CMPS promoter was isolated as a 404bp BamHI fragment from the plasmid pNOV4211 (see published International Patent Application No. WO01/73087; in particular SEQ ID NO: 3 and Example 14). This fragment was cloned into the vector pBluescript and screened for orientation. The resulting plasmid, pBS_CMPS, was digested with EcoRI/BamHI and the CMPS promoter fragment was cloned into pCR2.1-TOPO. The resulting plasmid, pTMR570, was used for cloning the promoter into pUC expression cassettes.

2.7 Cloning of the light chain expression cassette

The sequence encoding the light chain variable regions from the anti-K88 monoclonal antibodies, 36-41, 7-46 and 17-44, were synthesized chemically with optimized codon usage for expression in maize. A *Spe*I restriction site was introduced at the 5' end of the variable region and an *Xho*I restriction site was introduced at the 3' end of the variable domain in the highly conserved KLEIK motif of FR4 (SEQ ID NO.56). The SpeI/XhoI K88 VL domain fragments were cloned in a three way ligation with the K99 expression cassette fragments HindIII/XhoI and HindIII/SpeI from pTMT100, pTMR101and pTMR104 (see table 5). The CMPS promoter was cloned into the K88 36-41 expression cassette as described in Table 5.

2.8 Cloning of the heavy chain expression cassette

The sequence encoding the heavy chain variable region, from the anti-K88 antibody, and the heavy chain constant region, which was derived from a consensus sequence of mouse IgG1 (SEQ ID NO. 57) (Kabat et al., U.S. Dept Health and Human Services, U.S. Government Printing Offices 1987) was synthesized chemically with optimized codon usage for expression in maize. A *Spe*I restiction site was introduced at the 5' end of the variable region and an *Acc*I restriction site was introduced in the CH1 domain corresponding to amino acid 9. The SpeI/AccI K88 VH domain fragments were cloned in a three-way ligation using the K99 expression cassette fragments AccI/HindIII

and HindIII/SpeI from pTMR142, pTMR143 and pTMR144 {see table 5}. The CMPS promoter was cloned into the 36-41 expression cassettes as described in Table 5.

Table 5: Constructs containing K88 antibody elements.

Plasmids	Description	Vector	Construction
pTMR524	K88_36-41_VL (native)	pCR2.1	323 bp PCR product of 36-41 VL
pTMR525	K88_36-41_VH (native)	pCR2.1	393 bp PCR product of 36-41 VH
pTMR558	K88_36-41_VH Ubiquitin promoter + SEKDEL	pUC	MS 36-41 VH/Spe1/Acc1 (389 bp) ligated into pTMR142/Acc1/HinDIII (3,813 bp) and pTMR143/HinDIII/Spe1 (2,073 bp)
pTMR559	K88_36-41_VH Ubiquitin promoter	pUC	MS 36-41 VH/Spe1/Acc1 (389 bp) ligated into pTMR144/Acc1/HinDIII (3,794 bp) and pTMR143/HinDIII/Spe1 (2,073 bp)
pTMR560	K88_36-41_VH gZein promoter + SEKDEL	pUC	MS 36-41 VH/Spe1/Acc1 (389 bp) ligated into pTMR142/Acc1/HinDIII (3,813 bp) and pTMR142/HinDIII/Spe1 (3,516 bp)
pTMR561	K88_36-41_VH gZein promoter	pUC	MS 36-41 VH/Spe1/Acc1 (389 bp) ligated into pTMR144/Acc1/HinDIII (3,794 bp) and pTMR142/HinDIII/Spe1 (3,516 bp)
pTMR538	K88_36-41_VL Ubiquitin promoter + SEKDEL	pUC	MS 36-41 VL/Spe1/Xho1 (319 bp) ligated into pTMR100/HinDIII/Xho1 (2,065 bp) and pTMR101/HinDIII/Spe1 (3,516 bp)
pTMR539	K88_36-41_VL Ubiquitin promoter	pUC	MS 36-41 VL/Spe1/Xho1 (319 bp) ligated into pTMR104/HinDIII/Xho1 (3,181 bp) and pTMR101/HinDIII/Spe1 (2,065 bp)
pTMR540	K88_36-41_VL gZein promoter + SEKDEL	pUC	MS 36-41 VL/Spe1/Xho1 (319 bp) ligated into pTMR100/HinDIII/Xho1 (2,065 bp) and pTMR100/HinDIII/Spe1 (3,516 bp)
pTMR541	K88_36-41_VL gZein promoter	pUC	MS 36-41 VL/Spe1/Xho1 (319 bp) ligated into pTMR104/HinDIII/Xho1 (3,181 bp) and pTMR100/HinDIII/Spe1 (3,516 bp)
pTMR574	K88_36-41_VL CMPS promoter + SEKDEL	pUC	HindIII/BamHI (407 bp) CMPS promoter ligated with pTMR538 BamHI/AgeI (736 bp) and pTMR538 HindIII/AgeI (2841bp)
pTMR575	K88_36-41_VL CMPS promoter	pUC	HindIII/BamHI (407 bp) CMPS promoter ligated with pTMR539 BamHI/AgeI (717 bp) and pTMR539 HindIII/AgeI (2841bp
pTMR167	pTMR574+ pTMR571+ pTMR558	pNOV2117	HindIII/EcoRI (1,347bp) pTMR574 ligated to EcoRI/SpeI (483bp) pTMR571, SpeI/Acc65I (1,559bp) pTMR558 and pNOV2117/Acc65i/HindIII (9,158 bp)
pTMR168	pTMR575+ pTMR571+ pTMR559	pNOV2117	HindIII/EcoRI (1,329bp) pTMR575 ligated to EcoRI/SpeI (483bp) pTMR571, SpeI/Acc65I (1,541bp) pTMR559 and pNOV2117/Acc65i/HindIII (9,158 bp)
pTMR169	pTMR167 + pTMR568	pNOV2117	HindIII/AscI (1,364bp) pTMR167 ligated to MluI/Acc65I (3,627 bp) pTMR568 and pNOV2117/Acc65i/HindIII (9,158 bp
pTMR170	pTMR168 + pTMR569	pNOV2117	HindIII/AscI (1,345bp) pTMR168 ligated to MluI/Acc65I (3,609 bp) pTMR569 and pNOV2117/Acc65i/HindIII (9,158 bp
pTMR171	pTMR538 + pTMR167	pNOV2117	HindIII/MluI (2,940 bp) pTMR538 ligated to BssHI/Acc65I (2,026 bp) pTMR167 and pNOV2117/Acc65i/HindIII (9,158 bp
pTMR172	pTMR539 + pTMR571 + pTMR559	pNOV2117	HindIII/MluI (2,922 bp)pTMR539 ligated to BssHI/SpeI (467 bp) pTMR571, SpeI/Acc65I (1,541 bp) pTMR559 and pNOV2117/Acc65i/HindIII (9,158 bp
pTMR198	pTMR574 + pTMR571 +pTMR559	pNOV2117	HindIII/EcoR (1,347bp) pTMR574 ligated to EcoRI/SpeI (483bo) pTMR571, Spei/Acc65I (1,541bp) pTMR559 and pNOV2117/Acc65i/HindIII (9,158 bp
pTMR199	pTMR575 + pTMR571 +pTMR558	pNOV2117	HindIII/EcoR (1,329bp) pTMR574 ligated to EcoRI/SpeI (483bo) pTMR571, Spei/Acc65I (1,559bp) pTMR558 and pNOV2117/Acc65i/HindIII (9,158 bp
pTMR568	pTMR538 + pTMR558 (11114)	pNOV2117	pTMR558/Mlu1/Acc65i (3,626 bp) ligated to pTMR538/HinDIII/Mlu1 (2,940 bp) and pNOV2117/Acc65i/HindIII (9,158 bp)
pTMR569	pTMR539 + pTMR559 (11115)	pNOV2117	pTMR559/Mlu1/Acc65i (3,608 bp) ligated to pTMR539/HinDIII/Mlu1 (2,922 bp) and pNOV2117/Acc65i/HindIII (9,158 bp)
pTMR526	K88_7-46_VL (native)	pCR2.1	324 bp PCR Product of 7-46 VL
pTMR527	K88_7-46_VH (native)	pCR2.1	408 bp PCR Product of 7-46 VH

Plasmids	Description	Vector	Construction
pTMR562	K88_7-46_VH Ubiquitin promoter + SEKDEL	pUC	MS 7-46 VH/SpeI/AccI (404 bp) ligated to pTMR142/AccI/HinDIII (3,813 bp) and pTMR143/HinDIII/SpeI (2,073 bp)
pTMR563	K88_7-46_VH Ubiquitin promoter	pUC	MS 7-46 VH/SpeI/AccI (404 bp) ligated to pTMR144/AccI/HinDIII (3,794 bp) and pTMR143/HinDIII/SpeI (2,073 bp)
pTMR564	K88_7-46_VH gZein promoter + SEKDEL	pUC	MS 7-46 VH/SpeI/AccI (404 bp) ligated into pTMR142/AccI/HinDIII (3,813 bp) and pTMR142/HinDIII/SpeI (3,516 bp)
pTMR565	K88_7-46_VH gZein promoter	pUC	MS 7-46 VH/SpeI/AccI (404 bp) ligated into pTMR144/AccI/HinDIII (3,795 bp) and pTMR142/HinDIII/SpeI (3,516 bp)
pTMR544	K88_7-46_VL gZein promoter + SEKDEL	pUC	MS 7-46 VL/SpeI/XhoI (318 bp) ligated to pTMR100/HinDIII/XhoI (3,199 bp) and pTMR100/HinDIII/SpeI (3,516 bp)
pTMR545	K88_7-46_VL gZein promoter	pUC	MS 7-46 VL/SpeI/AccI (318 bp) ligated to pTMR104//HinDIII/XhoI (3,181 bp) and pTMR100/HinDIII/SpeI (3,516 bp)
pTMR542	K88_7-46_VL Ubiquitin promoter + SEKDEL	pUC	MS 7-46 VL/SpeI/XhoI (318 bp) ligated to pTMR100/HinDIII/XhoI (2,065 bp) and pTMR101/HinDIII/SpeI (3,516 bp)
pTMR543	K88_7-46_VL Ubiquitin promoter	pUC	MS 7-46 VL/SpeI/XhoI (318 bp) ligated to pTMR104/HinDIII/XhoI (3,181 bp) and pTMR101/HinDIII/SpeI (2,065 bp)
PTMR571	K88 7-46 VH CMPS promoter + SEKDEL	pUC	HindIII/BamHI (407 bp) CMPS promoter ligated with pTMR562 Acc65I/HindIII (2641 bp) and pTMR562 BamHI/Acc65I (1,634 bp)
PTMR572	K88 7-46 VH CMPS promoter	pUC	HindIII/BamHI (407 bp) CMPS promoter ligated with pTMR563 Acc65I/HindIII (2641 bp) and pTMR563 BamHI/Acc65I (1,616 bp)
pTMR566	pTMR542 + pTMR562 (11116)	pNOV2117	pTMR542/HinDIII/MluI (2,940 bp) ligated to pTMR562/Acc65I/MluI (3,641 bp) and pNOV2117/Acc65I/HinDIII (9,158 bp)
pTMR567	pTMR543 + pTMR563 (11117)	pNOV2117	pTMR543/HinDIII/MluI (2,922 bp) ligated to pTMR559/Acc65I/MluI (3,623 bp) and pNOV2117/Acc65I/HinDIII (9,158 bp)
pTMR528	K88_17-44_VL (native)	pCR2.1	PCR product of 17-44_VL (350 bp)
pTMR529	K88_17-44_VH (native)	pCR2.1	PCR product of 17-44_VH (363 bp)
pTMR546	K88_17-44_VH Ubiquitin promoter + SEKDEL	pUC	MS 17-44 VH/SpeI/AccI (393 bp) ligated to pTMR142/AccI/HinDIII (3,813 bp) and pTMR143/HinDIII/SpeI (2,073 bp)
pTMR547	K88_17-44_VH Ubiquitin promoter	pUC	MS 17-44 VH/SpeI/AccI (393 bp) ligated to pTMR144/AccI/HinDIII (3,794 bp) and pTMR143/HinDIII/SpeI (2,073 bp)
pTMR548	K88_17-44_VH gZein promoter + SEKDEL	pUC	MS 17-44 VH/SpeI/AccI (393 bp) ligated to pTMR142/AccI/HinDIII (3,813 bp) and pTMR142/HinDIII/SpeI (3,516 bp)
pTMR549	K88_17-44_VH gZein promoter	pUC	MS 17-44 VH/SpeI/AccI (393 bp) ligated to pTMR144/AccI/HinDIII (3,794 bp) and pTMR142/HinDIII/SpeI (3,516 bp)
pTMR550	K88_17-44_VL Ubiquitin promoter + SEKDEL	pUC	MS 17-44 VL/SpeI/XhoI (331 bp) ligated to pTMR100/HinDIII/XhoI (2,065 bp) and pTMR101/HinDIII/SpeI (3,516 bp)
pTMR551	K88_17-44_VL Ubiquitin promoter	pUC	MS 17-44 VL/SpeI/XhoI (331 bp) ligated to pTMR104/HinDIII/XhoI(3,181 bp) and pTMR101/HinDIII/SpeI (2,065 bp)
pTMR552	K88_17-44_VL gZein promoter + SEKDEL	pUC	MS 17-44 VL/SpeI/XhoI (331 bp) ligated to pTMR100/HinDIII/XhoI (2,065 bp) and pTMR100/HinDIII/SpeI (3,516 bp)
pTMR553	K88_17-44_VL gZein promoter	pUC	MS 17-44 VL/SpeI/XhoI (319 bp) ligated to pTMR104/HinDIII/XhoI (3,181 bp) and pTMR100/HinDIII/SpeI (3,516 bp)
pTMR554	pTMR546 + pTMR550 (11110)	pNOV2117	pTMR546/Acc65I/MluI (3,629 bp) ligated to pTMR550/HinDIII/MluI (2,952 bp) and pNOV2117/Acc65I/HinDIII (9,158 bp)
pTMR555	pTMR547 + pTMR551 (11111)	pNOV2117	PTMR547/Acc65I/MluI (3,611 bp) ligated to pTMR551/HinDIII/MluI (2,934 bp)) and pNOV2117/Acc65I/HinDIII (9,158 bp)

SEKDEL: C-terminal motif allowing ER-retention; MS: MS.

Native: original sequence cloned from hybridoma cells; when not specified, antibody sequences were codon-optimized for expression in plants. All codon-optimized sequences contained the g-zein signal sequence. The ubiquitin and the g-zein promoter were isolated from *Zea mays*. The CMPS promoter was isolated from Cestrum Yellow Leaf Curling Virus.

2.9 Transformation and Expression in Plants

Two independent antibody chains or antibody domains were expressed and assembled in plants using normal cellular processing. Antibody proteins were either targeted to the plant apoplast, or in the case of construct that have ER retention signal, targeted to organelles within the plant cell. Vacuolar targeting signals in the form of carboxyl-terminal propeptides are known in the art (Muntz, *Plant. Mol. Biol.*, 38:77-99, 1998; Neuhaus and Rogers, *Plant Mol. Biol.*, 38:127-44, 1998.

2.10 *Agrobacterium*-mediated transformation of maize

Transformation of immature maize embryos was performed essentially as described in Negrotto et al., *Plant Cell Reports* 19:798, 2000. For this example, all media constituents are as described in Negrotto et al., *supra*.

The genes used for transformation were cloned into a vector suitable for maize transformation. Vectors used in this example contained the phosphomannose isomerase (PMI) gene for selection of transgenic lines (Negrotto et al. *supra*).

Immature embryos, producing embryogenic callus were transferred to regeneration medium and grown in the light. After 2-3 weeks, plants were tested for the presence of the PMI genes and other genes of interest by PCR. Positive plants from the PCR assay were transferred to the greenhouse.

Trangenic corn plants expressing pTMR554, 555, 556 and 557 (Table 5) were regenerated. Antibody expression was analyzed by enzyme-linked immunoassay (ELISA, see Example 2.13) and by immunoblotting in several independent transgenic corn lines at stage V4-V6. ELISA analyses showed that transgenic corn plants expressed functional antibody and that the expression ranged between approximately 0.02 to 2% total soluble protein (TSP) (Table 6).

An Elisa assay was performed to test the binding capacity of the assembled antibody to the K88 antigen. The results are given in Table 6 and show that extracts from plants expressing the heavy and light chains reacted with the antigen. Levels of functional antibody, as determined by ELISA, reached up to 88% of total antibody molecule produced.

Typical results from Western blots of transgenic corn plants expressing antibody against K88 are shown in Fig. 4. No bands appeared on Western blots for non-transgenic corn plants under non-reducing and reducing conditions. Results under

reducing conditions are shown in Fig. 4A. Two major bands were present at about 50 and 25 kDa which was a similar size to the bands from the corresponding antibody purified from hybridoma cells. The upper band was identified as the heavy chain and the lower band was identified as the light chain by using a range of different detection antibodies.

All the independent clones produced bands of about 150 kDa that co-migrated under non-reducing conditions with the major band of the control antibody. This size most likely represented the fully assembled antibody. The mouse antibody purified from hybridoma cells yielded three bands under non-reducing conditions. Several other major bands with molecular weights between 50 and 135 kDa were also present in plant extracts. A range of probes was applied to Western blots of plant extracts to identify the antibody fragments. The bands of 150 kDa under non-reducing conditions bound with anti-Fc and anti-kappa probes, indicating that these molecules possessed the elements of a complete antibody. Interestingly, the pattern of bands seemed to differ for antibody molecules containing the ER retention signal. Also, the pattern varied between antibodies.

No extra bands were produced relative to untreated IgG1 after addition of the monoclonal antibody purified from hybridoma cells into corn plant extracts from non-transgenic plants. This observation indicates that the pattern of bands observed after Western blotting did not result from protein degradation due to tissue grinding and extraction.

The binding of the plant-produced antibody was confirmed by surface plasmon resonance. The dextran matrix of a CM5-rg sensorchip was coated with pili of *E.coli* strain K88 and after surface stabilization, a protein extract from corn leaves expressing the construct with or without the ER retention signal was injected. Similarly, antibodies purified from hybridoma cells were injected. Binding was observed in all cases. No binding was measured on a control surface lacking pili or when an extract from a non-transformed control plant was used. Functionality of plant produced purified antibodies was compared to the hybridoma produced monoclonal antibodies. Near identical thermodynamic (i.e. KD: 0.03 nM) and kinetic profiles are observed between hybridoma- and plant- produced proteins.

Monoclonal antibodies purified from plant material displayed the same binding affinities for the K88 antigen as the native IgG1 molecules isolated from murine

hybridoma material. Only two of the three plant-produced antibodies, 36/41 and 17/44, were purified from plant material and analyzed by surface plasmon resonance experiments. Plant-produced antibodies were extracted and purified from corn plant material as described herein. Antibody 36/41 bound the K88 surface with nearly identical kinetic characteristics as the hybridoma-produced antibody (Fig. 5). Similar results were obtained for antibody 17/44.

Table 6 Expression of K88 antibody genes in T0 corn plants.
Total soluble protein was isolated from young corn plantlets containing K88 antibody genes retained in the ER (pTMR554, event 11114) or targeted to the apoplast (pTMR555, event 11115). Levels of whole antibody and functional antibody were quantified by ELISA using a rabbit anti-mouse IgG1 and are indicated as percentage of total soluble protein.

Sample-extract	Antibody as % of TSP	Functional antibody as % of total antibody
Event 11114-1	0.61%	41.5
Event 11114-2	0.80%	47.0
Event 11115-1	2.64%	46.8
Event 11115-2	0.35%	73.6
Event 11115-3	0.37%	29.3
Event 11115-4	1.02%	88.0
Event 11115-5	0.70%	49.6
Control Leaf	-	

2.11 *Agrobacterium*-mediated transformation of rice callus

The rice (*Oryza sativa* var. japonica) cultivar "Kaybonnet" was used for generating transgenic plants. However, other rice cultivars can also be used (Hiei et al., *Plant J.*, 6:271-282, 1994; Dong et al., *Mol. Breeding*, 2:267-276, 1996; Hiei et al., *Plant Mol. Biol.*, 35:205-218, 1997). Also, various media constituents described below may be either varied in quantity or substituted. Embryogenic cultures were established from mature embryos by culturing on MS-CIM medium (MS basal salts, 4.3 g/liter; B5 vitamins (200 x), 5 ml/liter; sucrose, 30 g/liter; proline, 500 mg/liter; glutamine, 500 mg/liter; casein hydrolysate, 300 mg/liter; 2,4-D (1 mg/ml), 2 ml/liter; adjust pH to 5.8 with 1 N KOH; Phytagel, 3 g/liter). Established culture lines were inoculated and co-cultivated with the *Agrobacterium* strain LBA4404 (Cangelosi et al., *Meth. Enzymol.*, 204:384, 1991) containing the desired vector construction. *Agrobacterium* was cultured from glycerol stocks on solid 2YT medium (16 g Bacto-tryptone. 10 g Bacto-yeast extract, 5 g NaCl) (100 mg/L spectinomycin) for 3 days at 28 °C, then streaked again

and cultured for 1-2 days. *Agrobacterium* was re-suspended in liquid MS-CIM medium. The *Agrobacterium* culture was diluted to an OD_{600} of 0.2-0.3 and acetosyringone was added to a final concentration of 200 μM. *Agrobacterium* was induced with acetosyringone for at least 30 min before mixing the solution with the rice cultures. For inoculation, the cultures were immersed in the bacterial suspension for 30 min. The liquid suspension was removed with a vacuum aspirator and the inoculated cultures were placed on a Whatman filter paper on co-cultivation medium MS-CIM-As (MSB-CIM with 200 μM acetosyringone) and incubated at 22°C for two days. The cultures were then transferred to MS-CIM medium with Timentin (400 mg/liter) to inhibit the growth of *Agrobacterium*. For constructs utilizing the PMI selectable marker gene, cultures were transferred to selection medium containing mannose as a carbohydrate source (MS with 2% Mannose, 300 mg/liter timentin) after 7 days, and cultured for ~21 days in dark. Resistant colonies were then transferred to regeneration induction medium (MS with no 2,4-dichlorophenoxyacetic acid, 0.5 mg/liter IAA, 1 mg/liter zeatin, 200 mg/liter timentin 2% mannose and 3% sorbitol) and grown in the dark for 14 days. Proliferating colonies were then transferred to another round of regeneration induction media and moved to the light growth room. Regenerated shoots were transferred to GA7-1 medium (MS [Murashige and Skoog, *Plant Physiol.*, 15:473-497, 1962] with no hormones and 2% sorbitol) for 2 weeks and then moved to the greenhouse when they were large enough and have adequate roots. Plants were transplanted to soil in the greenhouse and grown to maturity.

Several transgenic plants were obtained. Antibody expression was analyzed by enzyme-linked immunoassay (ELISA) and by immunoblotting (see Example 2.13) of several independent transgenic rice plants. ELISA analyses showed that transgenic plants expressed functional antibody (Tables 7a, 7b & 7 C). Levels of functional antibody, as determined by ELISA, reached up to about 50% of total antibody molecule either targeted to the apoplast or residing in the ER.

Table 7a Expression of K88 antibody genes in T0 corn and rice plants.
Total soluble protein was isolated from corn and rice plants containing K88 antibody genes retained in the ER (pTMR554, event 11114; pTMR546, event 11110) or targeted to the apoplast (pTMR555, event 11115; pTMR547, event 11111). Levels of whole antibody and functional antibody were quantified by ELISA using a rabbit anti-mouse IgG1 and are indicated as percentage of total soluble protein.

# Plant analyzed	Plasmid	Description	% Total Antibody/TSP		% Functional Antibody	
			Median	Expression Range	Median	Expression Range
Maize						
59	11114	K88_3641 + ER	0.07	0.02 - 0.22	0.027	0.009 - 0.116
67	11115	K88_3641	0.08	0.01 - 0.51	0.026	0.004 - 0.418
9	11110	K88_1744 + ER	0.08	0.06 - 0.23	n.d	n.d
4	11111	K88_1744		0.03-0.94		
Rice						
41	11114	K88_3641 + ER	0.20	0.08 - 2.58	0.15	0.02 - 3.13

Table 7b Expression of K88 antibody genes in corn seeds. Total soluble protein was isolated from corn seeds containing K88 antibody genes retained in the ER (pTMR554, event 11114) or targeted to the apoplast (pTMR555, event 11115). Levels of whole antibody and functional antibody were quantified by ELISA using a rabbit anti-mouse IgG1 and are indicated as percentage of total soluble protein (TSP).

# Plant analyzed	Plasmid	Description	% Total Antibody/TSP		% Functional Antibody/TSP	
			Median	Expression Range	Median	Expression Range
Maize						
12	11114	K88_3641 + ER	0.6	0.01-2	0.4	0.01-2
30	11115	K88_3641	0.15	0.01-1	0.5	0.01-1.2

Table 7c Expression of K88 antibody genes in rice callus.
Total soluble protein was isolated from stable transformed rice callus containing K88 antibody genes retained in the ER (pTMR568 event 11114, pTMR167, pTMR169, pTMR171), targeted to the apoplast (pTMR569 event 11115, pTMR168, pTMR170, pTMR172) or where the light chain was retained in the ER and the heavy chain was targeted to the apoplast (pTMR198) or vice versa (pTMR199). Levels of whole antibody and functional antibody were quantified by ELISA using a rabbit anti-mouse IgG1 and are indicated as percentage of total soluble protein (TSP). All constructs contain the K88 36-41 VL and VH domains.

# Plant analyzed	Plasmid	Description	% Total Antibody/TSP		% Functional Antibody/TSP	
			Median	Expression Range	Median	Expression Range
Rice						
14	11114	Ubi VL+ :: Ubi VH +	0.87	0.18-1.80	0.59	0.23-1.20
15	11115	Ubi VL :: Ubi VH	0.63	0.17-2.53	0.22	0.10-1.64
15	pTMR167	CMPS VL+ :: CMPS VH +	0.99	0.17 - 2.11	0.60	0.09-1.76
15	pTMR168	CMPS VL :: CMPS VH	0.8	0.24 - 2.82	0.40	0.15-1.74
15	pTMR169	CMPS VL+ :: Ubi VH +	0.5	0.19 - 1.05	0.26	0.11-0.66

# Plant analyzed	Plasmid	Description	% Total Antibody/TSP		% Functional Antibody/TSP	
			Median	Expression Range	Median	Expression Range
15	pTMR170	CMPS VL :: Ubi VH	0.83	0.39 - 3.03	0.56	0.21-2.70
15	pTMR171	Ubi VL+ :: CMPS VH +	2.15	0.92 - 6.65	1.43	0.57-3.96
15	pTMR172	Ubi VL :: CMPS VH	n.d.	n.d.	n.d.	n.d.
14	pTMR198	CMPS VL+ :: CMPS VH	0.44	0.12 - 2.06	0.33	0.10-3.18
14	pTMR199	CMPS VL :: CMPS VH +	0.74	0.11 - 1.29	0.83	0.10-1.69

2.11 Protein extraction from plant tissues

Soluble protein extracts were prepared from rice and corn transgenic and non-transgenic callus, leaves and seeds. Samples of approximately 7 x 25 mm for leaf, 250 mg for seeds and 100 mg for callus were collected and placed into wells of 96-well plates (Corning, Corning, NY) containing 1 mm glass beads (BioSpec Products, Bartlesville, OK). The plates were kept on dry ice. Tissues were ground by an adapted reciprocating saw for 30 sec, rotated 180°, and ground for another 30 sec. After addition of cold protein extraction buffer (PEB: 50 mM Tris-HCl, 100mM KCl, 5mM EDTA, 0.1% Tween-20, pH 8.0) containing plant protease inhibitor cocktail mix (Sigma Chemical, St. Louis, MO, Cat# P9599), the tissues were ground once more and the plates were centrifuged at 4000 rpm for 10 min at 4°C. 1.8µl of PEB buffer containing 2% (v/v) protease inhibitor was used per mg of rice callus. Alternatively, tissues could also be frozen in liquid nitrogen, ground to fine powder in 1.5 ml tubes with a Teflon hand grinder, vortex, placed on ice with protein extraction buffer and processed as described above. The supernatants were used for ELISA and western blots analysis.

2.13 Expression analysis

The activity of the plant-expressed antibodies was determined by enzyme-linked immuno-assay (ELISA). The following buffers were used for ELISA: ELISA Diluent (11.9 mM Na_2HPO_4/NaH_2PO_4, 137 mM NaCl , 2.7 mM KCL, 0.05% Tween-20, 1% BSA, 3.3% NaN_3 , pH 7.4); 10x ELISA Wash Buffer (100mM Tris, 0.5% Tween 20, 3.3% NaN3, pH8.0); Blocking buffer: 5% (w/v) nonfat dry milk in ELISA diluent; Alkaline phosphatase substrate (Sigma Fast pNPP, Sigma Chemical, St. Louis, MO, cat #2770).

Microtiter plates (NUNC-MaxiSorb, Nalgene Nunc Intl. Rochester, NY) were coated with 250 ng per well of antigen in PBS buffer and incubated at 4°C overnight. The plates were blocked with 200 µl blocking buffer. Serial dilutions of transgenic plant extracts were prepared using extracts from untransformed plants. Aliquots of 25 µl of each diluted sample were transferred to the antigen-coated plates. Alkaline phosphatase-conjugated IgG Fc-specific rabbit anti-mouse IgG (Pierce Chemical, Rockford IL, cat. #31332) was added to each well at a 1:5000 dilution, followed by the addition of 50 µl P-nitrophenylphosphate as determined by the manufacturer (Promega, Madison, WI, USA). The reaction with the substrate was carried out for 1 h at 37°C. The absorption was determined at 405 nm using µQuant spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). All samples were run in duplicate. Protein concentrations were determined using the Bradford protein assay (BioRad, Hercules, CA).

For quantitative ELISA, plates were coated and incubated overnight at 4°C with 50 µl of 10 µg/ml rabbit, anti-Mouse (H+L) in PBS buffer (Zymed Laboratories, So. San Francisco, CA, Cat # 61-6500) or with rabbit anti-mouse IgG-kappa (Fizgerald, Concord, MA, cat # 41-RM28). Coated plates were blocked for 45 min at room temperature prior to the addition of 25 µl of diluted plant extracts and allowed to incubate at 37°C for 1.5 h. The rabbit, anti-mouse IgG (H+L) conjugated to alkaline phosphatase (Zymed Laboratories, Cat# 61-6522) was added at a 1:1000 dilution in ELISA diluent and incubated at 37°C for 1.5 h. The substrate reagent was added as previously described. Plates were washed three times between each step with the Elisa wash buffer. Transgenic protein extracts were diluted with extracts from wild-type tissue.

For Western blot analysis, equal amounts of protein, along with Mr standards (BenchMark, Invitrogen, Carlsbad, CA) were subjected to SDS-PAGE using precast 4-12% or tris-acetate 3-8% (w/v) NuPage gels (Invitrogen, Carlsbad, CA). Mouse IgG1 (Lampire Biological Laboratories, Pipersville, PA) or the purified monoclonal antibody from hybridomas were used as standards. Gels were electro-blotted onto nitrocellulose membranes (Invitrogen), and stained with Ponceau S (Sigma Chemical, St Louis, MO) to visualize the transfer. The blots were incubated in a blocking solution containing 10% (w/v) nonfat dry milk in TTBS (20mM Tris 7.5; 100mM NaCl; 0.1% TWEEN-20)

for 2 h at room temperature or overnight at 4°C. Detection of the whole antibody was performed by incubating the membrane with the rabbit, anti-mouse H+L (Zymed Laboratories, cat # 04-6600) at a dilution of 1:500 for 2 h, followed by incubation with the second antibody alkaline phosphatase-conjugated goat, anti-rabbit (Zymed Laboratories, Cat# 656122) (1:4000). Detection of the Fc fragment was performed by incubating the membrane with the alkaline phosphatase-conjugated anti-mouse Fc (Pierce Chemical, cat #31332) at a dilution of 1:2000. The light chain fragment was detected with rat, anti-mouse IgG-kappa (Zymed Laboratories, cat # 04-6600) at a dilution of 1:200, followed by alkaline-phosphatase goat, anti-rat H+L (Sigma Chemical, cat #A8438) at 1:5000. The blot was further washed as indicated above, and the immuno-labeled proteins were detected using the alkaline substrate following the manufacturer's recommendations (Promega, Madison, WI).

2.14 Conclusions

The experiments described above clearly demonstrate that expression of whole functional K88 antibodies was achieved in both maize and rice plant cells. Constructs containing ER retention signal on the heavy chain of the K88 antibody were found to have lower proportion of misassembled fragments (i.e. less dimers or trimers) and therefore more assembled functional antibody than other constructs without ER retention signals (see table 7c, construct 12645 vs 12646). Interestingly, constructs containing the ER retention signal lead to better levels of assembled antibodies that the equivalent construct without the ER retention signal (See Table 7c and compare 11114 vs 11115, 12639 vs 12640, 12641 vs 12642, CMPS and Maize ubiquitin promoters were used to express both heavy and light chains in different combinations. The combinations with highest effect of expression levels of the antibody and therefore preferred where those of the Maize ubiquitin promoter driving the expression of the heavy chain combined with the CMPS promoter driving the expression of the light chain or the CMPS promoter driving the expression of both heavy and light chains. Thus in one preferred arrangement, the expression of both chains is under the control of CMPS with ER signal retention signal in the heavy chain (12645 vs 12646). A second preferred arrangement is to express at least the heavy chain under the control of the CMPS promoter, preferably (though not necessarily) with the heavy chain being retained in the ER through the use of the ER retention signal.

Table 8 Sequence Identifiers for Light and Heavy Chain Variable Regions

Sequence Description	SEQ ID NO.
K99 VH codon optimized	1
K99 VH wild type	2
K99 VL codon optimized	3
K99 VL wild type	4
K88 17_44 VH-codon optimized	5
K88 17_44 VL codon optimized	6
K88 36_41 VH codon optimized	7
K88 36_41 VL codon optimized	8
K88 7_46 VH codon optimized	9
K88 7_46 VL codon optimized	10
K88 17_44 VH wild type	11
K88 17_44 VL wild type	12
K88 36_41 VH wild type	13
K88 36_41 VL wild type	14
K88 7_46 VH wild type	15
K88 7_46 VH wild type	16

Example 3

Antigen preparation

Antigen preparation and isolation procedures were based on the work of Karkhanis and Bhogal *Anal. Biochem.*, 155:51-55, 1986. For antigen preparation, 100 ml of Minca broth containing 1% IsoVitaleX (Becton Dickinson, Sparks, MD) in a 250 ml Erlenmeyer flask was inoculated with 50 µl of a 1:200 dilution of an OD_{600nm}=1.0 culture of *E.coli*. The culture was grown for 18 hours in a 37°C orbital shaker at 200 rpm and harvested after centrifugation at 8000 rpm in a Sorval SS-34 centrifuge for 20 min. The pellet was resuspended in 10 ml phosphate buffered saline (PBS), pH7.2, supplemented with 0.01% TWEEN-80, and then heated for 30 min in a 60°C water bath. The bacteria were homogenized with a polytron at top speed (28K rpm) for 5 min while on ice bath. After centrifugation at 15,000 rpm for 20 min, the supernatant was filtered through a 0.45µm membrane filter. A solution of 2.5% citric acid, pH 3.55, was

added to the filtered supernatant (pH ~7) to obtain a pH of 4.0. The pilus antigen was precipitated at 4°C for 30 min and then centrifuged for 20 min at 10,000 rpm in a Sorval SS-34 centrifuge. The pellet was resuspended in 1ml of 50mM phosphate buffer, pH7.2. The purity of the antigen was determined by running a non-denaturing 4-12% or 15% SDS-PAGE gel.

Example 4

Extraction and Purification of monoclonal IgG1

4.1 Hybridoma

Murine hybridoma cell cultures were grown to a maximum cell density of ~1-3 x 10^6 cells per ml and monitored for 2-3 days to allow for the expression and secretion of monoclonal IgG1. After this period, cultures were spun down at 1200 rpm for 8 minutes at room temperature. Supernatants were collected and passed through a 0.2 μm filter. The final supernatant solution consisted of DMEM (Dulbecco's Minimal Essential Medium with high glucose, Gibco, Invitrogen, Carlsbad, CA), 10% FBS (Sterile Fetal Bovine Serum, Sigma Chemical, St. Louis, MO), and 1X glutamine/Penicillin / Streptomycin (Gibco, Invitrogen, Carlsbad, CA). Supernatants containing mouse IgG1 antibody were applied directly to 5 ml HiTrap™ Protein G Columns (Amersham Biosciences, Piscataway, NJ) at 3 ml/min. Multiple passage of supernatants over the columns was unnecessary as >95% of all IgG1 material from each supernatant bound to the column on the first pass. Mobile phases consisted of 1X PBS (running buffer, Fisher Scientific, Pittsburgh, PA) and 0.1 M glycine pH 2.7 (elution buffer, Sigma Chemical). Antibody collections in 0.1 M glycine were diluted 20% (v/v) with 1 M TrisHCl, pH 9.0, for neutralization. IgG1 collections were pooled and dialyzed exhaustively against H_2O and subsequently 1X PBS. The dialysates were diluted 1:1 (v/v) with glycerol and stored at -20 °C. The presence of glycerol in diluted samples did not affect the binding affinity of any IgG1 antibodies as determined by Surface Plasmon Resonance analysis (Biacore, Piscataway, NJ). The concentration of each IgG1 stock solution was determined by Bradford analysis (Biorad, Hercules, CA) prior to glycerol addition using a commercial murine IgG1 stock solution (1 mg/ml - Sigma Chemical, St. Louis, MO, Cat#M9269) as a standard.

The binding characteristics of purified murine monoclonal antibodies from hybridoma cell cultures to the K88 pilus protein antigen (also known as the F4 fimbriae

adhesin) were analyzed by surface plasmon resonance (Biacore, Piscataway, NJ). The K88 pilus protein could not be used as the analyte in the analyses due to concentration dependent self-association. As an analyte, K88 exists in multiple, complex forms and binding to immobilized anti-K88 antibodies cannot be modeled. Sensible data could be achieved using K88 as ligand, however. Attachment of K88 at very low concentrations to the biosensor chips resulted in highly sensitive chip surfaces. Binding of monoclonal antibodies to the K88-linked surfaces fit well to a standard bivalent analyte model system:

$$A + B \underset{kd1}{\overset{ka1}{\leftrightarrow}} AB + B \underset{kd2}{\overset{ka2}{\leftrightarrow}} AB_2,$$

where A is the bivalent antibody free in solution, B is an interaction site on the surface of the chip, AB is a singly bound antibody molecule and AB_2 is the situation where both interaction sites of the antibody are involved in surface interactions. The baseline response level of K88-bound surfaces did not decrease over the lifetime of chip use. This result strongly suggests that all K88 molecules capable of binding IgG1 were covalently attached to the chip surface as opposed to noncovalently attached as multimeric subunits. Attachment of 50 RU of K88 antigen to the CM5 surface led to a maximum IgG1 response of approximately 130 RU regardless of the IgG1 used for the analysis. This R_{max} value is smaller than the 225 RU 'ideal' response level expected if K88 were a monomer (28 KDa) on the chip surface. This is unsurprising considering that surface linkage by random primary amines of the ligand often reduces the amount of active ligand the surface presents to potential analytes.

The three monoclonal IgG1 antibodies (17/44, 36/41 and 7/46) each displayed unique binding characteristics to the K88 antigen. In all cases, the overall association-dissociation rates of each antibody were dominated by k_{a1} and k_{d1} with minimal contributions from k_{a2} and k_{d2}. "Pseudo" equilibrium dissociation constants, K_d, were found for each antibody based on k_{d1}/k_{a1} alone. Sensogram curves for each IgG1 are shown in Fig. 6. The kinetic parameters derived from fitting the curves to the bivalent analyte model are given in Table 9. Monoclonal antibody 7/46 had the weakest affinity for K88 and 36/41 had the highest. Dissociation of 36/41 was so slow that an extra experiment was performed to track the signal decay (release of 36/41 IgG1) over a 3.5 hour period where some dissociation could actually be measured. The residuals for the

bivalent analyte fits are all randomly clustered about zero. A slight deviation from the bivalent analyte model is apparent in 17/44 indicating the possibility of multiple binding sites for 17/44 on the K88 surface or potentially non-ideal behavior of the IgG1 molecule in solution.

Table 9 Rate constants derived from Bivalent analyte fits to multiple concentrations of each of the three IgG1 antibodies (see Fig. 6). Pseudo K_D refers to k_{d1}/k_{a1} with the assumption that k_{d2} and k_{a2} do not contribute significantly to the observed rates.

IgG1 identity	k_{a1} (1/M*s)	k_{a2} (1/ RU*s)	k_{d1} (1/ s)	k_{d2} (1/ s)	Pseudo K_D
7/46	$3.4*10^5$	$5.4*10^{-4}$	$7.5*10^{-3}$	$1.2*10^{-2}$	20 nM
36/41	$9.6*10^5$	$4.8*10^{-3}$	$1.1*10^{-5}$	$2.1*10^{-1}$	10 pM
17/44	$5.4*10^5$	$2.9*10^{-3}$	$3.0*10^{-4}$	$2.6*10^{-2}$	600 pM

4.2 Extraction and purification of plant-produced antibody

Frozen transgenic corn material (both corn leaf and seed) was initially pulverized while submersed in N_2(l) using a SPEX CertiPrep Model 6800 freeze mill. Frozen powder was further pulverized using a mortar and pestle in the presence of N_2(l). Protein was extracted from pulverized material with an aqueous 25 mM TrisHCl, 100 mM KCl, 5 mM EDTA, 0.1 % TWEEN-20 (polyoxyethylene-sorbitan monolaurate) buffer including a 1:10000 dilution of a concentrated protease inhibitor cocktail (ICN Biomedicals, Costa Mesa, CA, #B000126). The dry mass:extraction buffer ratio was 1:4 and yielded total soluble protein concentrations of ~1.6 ± 0.6 mg/ml for corn leaf and 2.7 ± 0.4 mg/ml for corn seed. Plant extracts were centrifuged at 15000 rpm for 20 minutes and filtered with a 0.2 µm filter. Extracts were subsequently applied to 1 ml HiTrap™ Protein G Columns at a flow rate of 1 ml/min. Running buffer and elution buffer were 1X PBS and 0.1 M glycine, pH 2.7, respectively. Purified plant IgG1 was dialyzed against H_2O and subsequently against a 0.1 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.01% TWEEN-20 buffer (the running buffer used for Surface Plasmon Resonance analysis). The presence of plant produced murine IgG1 was confirmed by Western Blot analysis. Plant produced IgG1 concentrations were determined by Bradford analysis.

Example 5

Surface Plasmon Resonance

Proteins/ligands were covalently attached to Biosensor CM5 (BioSensor, Uppsala, Sweden) chip surfaces via their exposed primary amine groups using the immobilization protocols within the BIA3000 software and standard N-ethyl-N'-(dimethylaminopropyl)-carbodimide-hydrochloride/400 mM N-hydroxy-succinimide (NHS/EDC) activation chemistry. Unreacted surface sites were blocked with ethanolamine to prevent further reactivity. Immobilization levels are discussed in Example 4.1. Immobilization was achieved by addition of 0.5 to 10 µg/ml ligand in 10 mM acetate, pH 4.0. Experiments designed for quantitative determination of ligand/analyte kinetic and affinity parameters were performed at a flow rate of 50 µL/min, 40 °C. Experiments designed for qualitative comparison of multiple analytes were performed at a flow rate of 25 µL/min, 25 °C, to conserve protein. Running buffer contained 0.1 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.01% TWEEN-20, pH 7.4. Reference flow cells were activated with NHS/EDC and blocked immediately with ethanolamine.

Example 6

Intestinal Levels of Orally Administered Monoclonal Antibodies

Fifteen barrows (PIC, C_{15} x Canabrid) were used. Pigs were surgically fitted with a simple T-cannula at the distal ileum. Pigs weighed approximately 30 kg and were approximately 9 weeks old at the start of the experimental period. Approximately five pigs were cannulated each day and allowed a post-operatory recovery period of 3-7 days.

The pigs were housed in metabolic pens measuring 1.2 m (length) x 0.8 m (height) x 1.2 m (length) in one room. The pens allowed freedom of movement of the pigs during the entire experiment. Metabolism pens were fitted with a bowl feeder and bowl type drinker, and a plexi-glass panel mounted between adjacent pens allowing pigs to have visual contact.

After pigs recovered their appetite, three meals of equal weight were provided (~1:1) at ~08:30, 11:30, and ~15:30. Pigs had free access to fresh water at all times. The study design was a randomized complete block design (see Table 10). Pigs were blocked by day of surgery and by weight, and were randomly allocated to one of five

treatments. Individual pigs were considered the experimental unit. Personnel involved in the conduct of the analytical and/or quantitative laboratory assays on the ileal digesta and fecal samples were blinded or masked as to the experimental block or treatment provided to any of the pigs.

Table 10 Study design for Immunoglobulin treatment of Pigs

Group number	No of animals	Immunoglobulin treatment	Means of administration	Dose per treatment	Regimen
1	3	Purified bovine anti-K99 immunoglobulin	Dry- mixed in feed in combination with dry bovine colostrum	500 mg	Single bolus
2	3	Purified bovine anti-K99 immunoglobulin	Dry- mixed in feed	500 mg	Single bolus
3	3	Purified bovine anti-K99 immunoglobulin	Dry- mixed in feed	170 mg	3 X daily
4	3	Purified bovine anti-K99 immunoglobulin	Liquid- dissolved in water	500 mg	Single bolus
5	3	Purified bovine anti-K99 immunoglobulin	Liquid- dissolved in water	170 mg	3 X daily

On Day 0, pigs within a block were randomly allocated to one of the above five treatments. Treatments were administered once or three times a day (depending on the experimental group) for three consecutive days (days 0 through 2). On Day 1, pigs were fed three meals at approximately 8:00, 11:00, and 14:00. Plastic bags were attached to Velcro rings around the anus and to the ileal T-cannula allowing feces and ileal digesta to be collected throughout the day for analysis of bovine anti K99 antibodies and IgG1 levels. No chemical or preservative was added to the collection bags. Ileal digest and feces were also collected on Day 0, prior to administration of the first treatment with bovine immunoglobulin.

To ensure that pigs consumed all of the dry test articles, the test article was mixed in with an aliquot of feed (~100g), which was provided prior to a meal. Once that small amount of feed was consumed, the rest of the meal was administered. Pigs receiving the test article dissolved in water received it orally in a volume of 10 ml prior, to being fed. To determine the transit time, adsorption and/or decay rate of IgG1 and anti K99 antibodies, ileal digesta and feces were collected as per the Baseline period on Days 0 to 4.

During the post-operative recovery, baseline, treatment, and collection periods, animals were observed three times daily for health status evaluation and the amount and

consumption of each meal was verified and recorded. For the most part, no ill effects were associated with the administration of bovine immunoglobulin to pigs. One pig (group 5) was observed vomiting on Day 0, shortly after the administration of the first treatment. The availability of water was confirmed and recorded three times daily. Consumption or refusal of the test article was documented.

On Days 0 and 1, average antibody levels against K99 were highest in pigs receiving a single bolus (500 mg) of dry bovine immunoglobulin mixed with colostrum (group 1) three hours following treatment, followed by pigs treated with a dry single bolus (500 mg) mixed directly in the feed (group 2) (Figures 7 and 9). K99 antibody levels on day 2 were highest three hours following treatment in group 2, followed by group 1 (Figure 11), whereas anti K99 levels were considerably lower on days 0-2 in pigs treated with a single dose (500 ml) dissolved in water (Figures 7, 9, and 11). In all three of the single dose groups, anti K99 levels had dropped to significantly 6 hours following administration of immunoglobulin. Anti-K99 levels were approximately three times lower in groups receiving bovine immunoglobulin doses of 170 mg (both dry and dissolved in water) three times daily on all three treatment days (Figures 8, 10, and 12). In contrast with the single dose groups, anti K99 levels in pigs treated three times daily were sustained both at 3 and 6 hours following treatment. Similar results were observed when anti K99 levels for all treatment days were averaged (Figure 13).

Total IgG1 detection in ileal content was variable. In several instances, IgG1 levels in one pig treated with a single bolus (500 mg) of the dry immunoglobulin mixed in with colostrum (group 1) were much higher than those in the other 2 pigs of the same group, resulting in a significantly higher average than that obtained in other groups (Figures 14-19). Similar results were observed when IgG1 levels for all treatment days were averaged (Figure 20). The levels of anti K99 antibodies and total IgG1 in feces were below the detection level the assays used.

Example 7

Optimation of Antibody C_H3 Domain

7.1 Residue frequency analysis of IgG Fc domain (CH2CH3)

A total of 36 unique IgG Fc sequences were collected by performing the Tera-Blast™ routine on the nonredundant protein sequence data set maintained by NCBI

(The National Center for Biotechnology Information) and using the bovine, murine and human Fc sequences as bait. All sequences utilized for the Fc residue positional frequency analysis were at least 95% unique (i.e. at least 10 residue variations out of ~220 residues from any other sequence within the data set). Without the 5% cutoff, the sequence set burgeons to over 100 sequences. The redundancy creates considerable bias and was therefore eliminated. Fig. 22A displays a portion of the Clustal W sequence alignment for all sequences in the data set.

Positional frequency and positional entropy calculations were performed on the 36 sequences utilizing a *Perl* program written in-house. The program uses ClustalW sequence alignment files as input (Higgins et al., *Nuc. Acids Res.*, 22:4673-4680, 1994 - http://www.ebi.ac.uk/clustalw). The residue frequency, $p_i(r)$, for each position, i, in an individual sequence is simply the number of times that particular residue-type (r = A,C,D...V,W,Y) is observed within the data set divided by the total number of sequences. The Positional Entropy, $N(i)$, was calculated as a measure of every residue position's variability (Shenkin et al., *Proteins*, 11:297-313, 1991). The Positional Entropy is a function of the Shannon informational entropy, $H(i)$:

$$N(i) = e^{H(i)}, H(i) = -\sum_{r=A}^{Y} p_i(r)\ln(p_i(r)).$$

Scores for each sequence were crudely calculated as a function of their similarity to the 'optimal' or consensus sequence (Table 11). Interestingly, the human sequence was closest to the consensus regardless of whether any other primate sequence was included in the overall data set. The bovine sequence scored moderately while the mouse score was low. The variation in scores likely reflects underlying biases in the overall data set rather than some phenomenon where the human sequence is most highly evolved to be closest to the optimal. Even taking the biasing into account, the murine IgG1 sequence in particular scores low and is fairly distinct from other Fc sequences, including murine IgG2.

Data from the analysis was used to direct mutagenesis for the optimization of the bovine CH3 domain. Mutations were selected based on four criteria. First, the individual sequence's residue frequency at a given position divided by the most common residue frequency at the same position for the entire data set must be less than 0.2 (i.e. rf/mfr $\leq$0.2). Second, the mutation must be conservative based on CH3 structure analysis and residue type; thus, no charge reversals were made and so forth.

Third there must be an absence of covariation at this position with other residue positions throughout the sequence. Forth, the native residue to be mutated must not be conserved within the individual species IgG subclass (bovine in the case of the present example). This final criterion is related to the no-covariation criterion.

Several CH3 domains, including bovine CH3, contain insertions or deletions; therefore, the Fc data set was chosen for residue numbering throughout the manuscript. Numbers in parentheses refer to the standard full-length human IgG residue numbering while the numbers directly next to the residue letter refer to numbering originating from the Fc data set.

Table 11 Sequence scores reflecting the similarity to the consensus sequence for individual sequences of the IgG data set. The maximum score is 238 for a complete match of all Fc residues of an individual sequences with the 238 residue positions of the consensus sequence. Numbers in the parentheses represent the number of related species from a given Family represented in the data set. Families are arbitrarily broken down into primates, rodents, livestock (i.e. cattle, sheep, horses, goats), domestic animals (cats and dogs) and others. *Rhesus monkey was not included in the data set in this case, but compared against it.

Species-IgG1	Full Data Set Scores	Trimmed Data Set Scores	Primate unbiased Data Set Scores
Human	223.7 (9)	223.0 (4)	216.4 (1)
Rhesus Monkey	217.3 (9)	217.7 (4)	209.0 (1)*
Bovine	205.6 (11)	208.4 (4)	212.2 (4)
Rabbit	201.6 (12)	204.0 (7)	204.6 (7)
Cat	201.1 (5)	204.2 (3)	206.1 (3)
Dog	196.8 (5)	197.6 (3)	200.3 (3)
Hamster	191.0 (12)	194.3 (3)	195.5 (7)
Mouse	189.0 (12)	193.7 (7)	196.7 (7)
Rat	185.5 (12)	189.0 (7)	191.6 (7)
Echidna	135.7 (12)	146.0 (2)	150.4 (2)

7.2 Expression and Purification of Bovine, Murine and Human CH3 domains

The human CH3 domain was subcloned from genbank source #BC014258 purchased from the ATCC (Manassas, VA). Bovine CH2CH3 and CH3 constructs were subcloned from bovine constructs derived from a bovine spleen cDNA library purchased from Stratagene (La Jolla, CA). Murine CH3 was subcloned from a synthetic murine IgG1 gene based on the murine Kabat database consensus sequence. CH3 PCR inserts and commercial pET21a plasmid (Novagen, Madison, WI) were digested using the designed BamHI and XhoI restriction sites (enzymes from Invitrogen, Carlsbad, CA). Inserts were ligated into the vector using the H.C. T4 DNA ligase (Invitrogen).

The QuickChange® Site-Directed Mutagenesis kit (Stratagene) was used to create individual mutations in the bovine CH3 pET21a vector.

The following primers were used to amplify the constant domain fragments from full-length antibody gene sequences. Numbers in parentheses indicate the total number of nucleotides for each amplified polynucleotide fragment.

Human C_H3 (330):

5'-gaattaaggatccaaagccaaaggccagccccgcgaacc-3' (SEQ ID NO. 58)

5'-tttattgattattgctcgagtttacccggagacaggga-3' (SEQ ID NO. 59)

Murine C_H3 (330):

5'-aattaatgaattaaggatccaagaccaagggccgcccgaagg-3' (SEQ ID NO. 60)

5'-tttattgattattgctcgagcttgcccggggagtgagagagg-3' (SEQ ID NO. 61)

Bovine C_H3 (336):

5'-aattaatgaattaaggatcccgcaccaaaggccctgcc-3' (SEQ ID NO. 62)

5'-tttattgattattgctcgagcttgccggcggacttggagg-3' (SEQ ID NO. 63)

Bovine C_H2C_H3 (642):

5'-ttaatgaattaaggatccggcggcccatctgtgttcatcttc-3' (SEQ ID NO. 64)

5'-tttattgattattgctcgagcttgccggcggacttggagg-3' (SEQ ID NO. 65)

The BL21(DE3), BL21(DE3-trxB) and Rosetta-Gami *E. coli* cell lines (all from Novagen) were investigated as potential hosts for protein expression. Cells from all three hosts were grown at 37 °C in luria broth (LB) following transformation/inoculation. Cell production was allowed to reach an A_{600} reading of 0.6-0.8 AU before induction with 1 mM IPTG (isopropyl β-D-thiogalactoside). Protein expression was carried out at 37 °C for 5 hours. Cells were harvested by centrifugation at 5000g and cell pellets were frozen overnight.

Cell pellets from 1 L cultures were resuspended in 30 ml 8 M urea, pH 8.5. Resuspensions were sonicated, centrifuged for 20 minutes at 15000 rpm and passed through 0.2 μM filters. Initial purifications were performed by His-Tag affinity chromatography. Crude protein suspensions were applied to Ni^{2+}-NTA agarose (Qiagen, Valencia, CA) gravity columns. Relatively pure CH3 constructs were eluted from the affinity column with 8 M urea, pH 4.4. Semi-pure proteins were further purified by reverse phase HPLC on a Jupiter C5 column (Phenomenex, Torrance, CA) using H_2O/Acetonitrile (0.1%TFA) gradients. The HPLC system consisted of a Dionex

(Sunnyvale, CA) GP50 quadrapump unit linked to an AD25 absorbance detector and a LC10 manual injector. Pure proteins were frozen, lyophilized and resuspended at neutral pH and stored at 4 °C.

The sequences of the three proteins are shown in Fig. 22B aligned against the consensus sequence derived from the residue frequency analysis. Bovine CH3 (bCH3) was used to scout for the most suitable expression host. The BL21(DE3), BL21(DE3-trxB) and Rosetta-Gami *E. coli* cell lines (all from Novagen, Madison, WI) were investigated as potential hosts for protein expression. Constructs were developed for cytoplasmic protein expression (i.e. lacking signal peptides directing the protein to the periplasmic space). bCH3 expresses well in all hosts and all hosts yielded a similar amount of soluble protein (~70% soluble/30% insoluble based on reverse phase HPLC analysis).

CH3 contains a single disulfide bond between C170(367) and C230(425). The BL21(DE3) host yielded little oxidized protein (< 20% based on acrylamide gel analysis). Both the BL21(DE3-trxB) and the Rosetta-Gami cell lines produced significant oxidized bovine CH3 (between 70-80% oxidized). The Rosetta-Gami growth phase was consistently longer and required the use of 4 separate markers making its handling more cumbersome. Therefore, the BL21(DE3-trxB) line was chosen for the expression of all CH3 constructs discussed in this example although other expression hosts can be used. Initial purification of the CH3 domain was performed using Ni^{2+}-NTA gravity columns (Qiagen, Valencia, CA). It was determined that bovine CH3 could be refolded in native buffer systems; therefore, all cell pellets were solubilized in 8 M urea at pH 8.5 prior to sonication to capture both the ~70% soluble and ~30% insoluble protein fractions and purified as described above. As demonstrated by HPLC,oxidized CH3 eluted significantly before reduced CH3 and could, therefore, be separted into purely oxidized and reduced forms, Fig. 23A. Purity of the CH3 domains is shown in Fig. 23B. Final yields of bCH3 ranged from 20-40 mg pure protein/1L shaker flask culture. Interestingly, murine CH3 (mCH3) did not produce significant amounts of oxidized protein and human CH3 (hCH3) expression yielded almost none. Enough oxidized mCH3 could be collected for biophysical analysis, while hCH3 collections all appeared to be at least partially reduced, Fig. 23B. bCH3 and mCH3 ran as dimers on a BioSpec2000 (Pharmacia) gel filtration column. hCH3 eluted in several forms, predominately trimer, dimer and monomer; another indication that it is not

wholly folded/oxidized. The proteins were expected to run as dimers based on their known biophysical behavior.

7.3 Bovine and Murine CH3 are stably and cooperatively folded

Circular dichroism (CD) and fluorescence measurements were performed using an Aviv Model 215 spectrometer equipped with a thermoelectric cuvette holder and the total fluorescence accessory. CD spectra were obtained by scanning at 1 point/nm. All final spectra were the average of at least 4 scans utilizing a signal averaging time of 2 s/λ. Background spectra were subtracted from sample spectra and the resulting corrected spectra were converted to mean residue ellipticity (deg cm^2 $dmol^{-1}$) based on the protein concentration and the number of peptide bonds. Temperature melts from 1 to 101 °C (2 °C temperature steps) were monitored separately using fluorescence, near UV CD and far UV CD. The fluorescence excitation wavelength was 278 nm with a 3nm bandwidth; the near UV CD signal was monitored at 278 nm using a 2 nm bandwidth; and the far UV CD signal was monitored at 217 nm using a 1 nm bandwidth. Signal averaging times for all melting experiments were 50 s/°C. A CD transparent buffer was used for all experiments (2 mM phosphate, borate, citrate, 10 mM NaCl, pH 7.5). The melting temperature of bovine and murine CH3 did not vary with concentration, therefore the curves were fit to a two-state/single-molecule unfolding model:

$$F(folded) \Leftrightarrow U(unfolded), \text{ where } K_u = [U]/[F]; K_u = f_u/(1-f_u)$$

to obtain precise T_m values. [U] and [F] are the molar concentrations of the unfolded and folded states, respectively, f_u is the fraction of protein in solution that is unfolded. According to the Gibbs-Helmholtz equation,

$$K_u = e^{-\Delta G_U^\circ(T)/RT}, \Delta G_U^\circ(T) = \Delta H_U^\circ(T_m) * (1 - T/T_m) - \Delta C_p^\circ * [(T_m - T) + T\ln(T/T_m)].$$

Assuming that the signals of the folded and unfolded states vary linearly with temperature and that the heat capacity difference, ΔC_p°, between the folded and unfolded states is constant over the temperature range of the experiment, the melting curves can be fit to the following equation:

$$\theta(T) = \frac{d + c*T + (b + a*T)e^{-\Delta G_U^\circ(T)/RT}}{1 + e^{-\Delta G_U^\circ(T)/RT}},$$

where (d + c*T) and (b+a*T) describe the linear changes with temperature for the signals of the folded and unfolded states, respectively. A theoretical ΔC_p°, 1727 cal/mol*K, was calculated based on an estimate of the change in accessible surface area (ΔASA) between the folded and unfolded states of a protein (Myers et al., *Prot. Sci.*, 4:2138-2148, 1995). ΔASA scales with the size of a protein and was estimated to be 9323 Å^2 for the CH3 monomer. The ΔASA of CH3 dimerization has been calculated previously, 1090 Å^2 (Miller, *J. Mol. Biol.*, 216:965-973, 1990). The sum of these two values was used to calculate ΔC_p° between the folded and unfolded states.

Gel filtration was performed on a BioSpec2000 (Amersham Pharmacia, Piscataway, NJ) HPLC gel filtration column. Molecular weight standards included bovine serum albumin, carbonic anhydrase, lysozyme and ubiquitin. Typically, between 10-100 μg protein was injected per run. Runs were performed at 1 mL/min using 0.1 M HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.5 as the mobile phase.

The CH3 domains all contained significant secondary structure as judged by their circular dichroism (CD) spectra, Fig 24A. bCH3, mCH3 and hCH3 all exhibited a minimum at 217 nm, characteristic of β-sheet structures. This is consistent with the antibody fold being composed of a two-fold β-sheet sandwich structure, Fig 21. The hCH3 spectrum was significantly different in intensity and shape from the spectra of bCH3 and mCH3, but was very similar to what is observed for reduced bCH3 (data not shown). The bCH3 and mCH3 spectra were nearly superimposable indicating their structures must be near identical. This was not entirely expected considering that bCH3 and mCH3 are only 54% identical in sequence to one another. Local structural rearrangements that affect the CD spectra might be expected in order to accommodate the differences in primary sequence.

Temperature induced unfolding of the domains was studied by monitoring the far UV CD signal at 217 nm, the near UV CD signal 278 nm and fluorescence using an excitation wavelength of 278 nm. bCH3 and mCH3 both undergo a single unfolding transition with midpoints (T_m) at 349.5 ± 0.5 K and 346.8 ± 0.5 K, respectively. Refolding of both bCH3 and mCH3 was almost completely reversible. The CD spectra at 5 °C before and after temperature denaturation were virtually unchanged. hCH3 did

not display a two-state unfolding behavior Fig. 24B. At approximately 340 K, the CD and fluorescence signal of hCH3 begins to change dramatically, but not in a single, sigmoidal fashion. This behavior was also observed for purified reduced bCH3 (data not shown). Additionally, the CD spectrum of hCH3 after temperature denaturation does not completely return to its original shape, but is diminished in intensity.

The T_m of both bCH3 and mCH3 was unaffected by concentration. Fluorescence measurements were performed using 0.5 and 4 μM bCH3, near UV CD measurements were performed with 10 μM bCH3 and far UV CD measurements with 30 μM bCH3 (See Fig. 24B for far UV CD temperature denaturation). Over this 60-fold range of concentrations, no change in the T_m was observed. This result indicates one of two possibilities. The first possibility is that the dimer dissociates before the unfolding transition occurs. The second possibility is if the K_D is a low pM or fM, perhaps no free CH3 exists to push the stability of the folded dimer up at high concentrations. Also, the folding rate of the protein may be unaffected by the rate of dimer association, but instead dominated by monomer folding as observed by Buchner and coworkers (Thies et al. *J. Mol. Biol.*, 293:67-79, 1999). In any case, the stability of bCH3 and mCH3 are very similar, despite their differences in primary sequence.

7.4 Directed Mutation of Bovine CH3 Based on Residue Frequency Analysis Allows for the Stabilization of the Domain.

Five potentially non-ideal residue sites were identified when pitting the bovine CH3 sequence against the Fc data set, Fig. 22B. The sites are S174(371), Y179(376), G197(392), S207(402) and T246(441) where the numbers in parentheses are the standard human IgG residue numbering. Each residue site is distant from the four other sites based on the structure of hCH3 (DeLano et al. *Science*, 287:1279-1283, 2000) and is thus expected to have an independent affect on the structure and stability of the domain. The following point mutations were made in an attempt to 'optimize' bCH3: S174G, Y179D, G197K, G197A (conservative mutation), S207G and T246L.

All mutant proteins display near identical CD spectra to that of the native protein (Fig. 25A) indicating no major structural changes were induced by any of the point mutations. The temperature unfolding transitions of all six mutants were monitored by near and far UV CD at 278 and 217 nm, respectively. Each mutant CH3 domain

exhibited a unique T_m, reproducible by both near and far UV CD. Temperature denaturation results for all constructs are given in Table 12.

Table 12 Results of fitting the temperature denaturation curves of bCH3 and the 6 mutant proteins to a two-state unfolding model. *Indicates hypothetical triple mutant assuming that individual mutant stabilizations are additive to overall protein stability.

Construct	T_m (K)	ΔT_m (K)
bCH3	348.8 ± 0.5	-
S174G	349.1	0.3
Y179D	349.6	0.8
G197K	354.2	5.4
G197A	351.7	2.9
S207G	353.2	4.4
T246L	352.3	3.5
*G197K/S207G/T246L	*361.1	*13.3

The G197K, S207G and T246L mutations all had a stabilizing affect on bCH3. Previous studies using residue frequency analysis to optimize protein stability have shown that such mutational increases in stability are generally additive (Roth and Davidson, *Protein Sci.* 9:2457-2469, 2000, Steipe et al. *J. Mol. Biol.*, 240:188-192, 1993). Unfolding curves of bCH3, the 6 mutant CH3 domains and a hypothetical triple mutant are shown in Fig. 25B.

Example 8

Optimization of Murine and Human C_H2C_H3 and Bovine C_H2

Based on the mutational criteria established for the bovine C_H3 domain, potentially stabilizing mutations within the C_H2 domain of bovine IgG1 and within the C_H2C_H3 domains of murine and human IgG1 were identified using the same data set and residue frequency analysis. Due to stringent mutational selection, none of the mutants within the bovine C_H3 had a destabilizing influence on the domain. In fact, the worst performing mutations, Y179D and S174G, resulted in no change in the overall stability of the domain. This provides a high degree of confidence that the mutations within bovine C_H2 and murine and human C_H2C_H3 that are given below will have a beneficial affect on the stability of each domain.

The data set consensus sequence is shown below with the Fc (SEQ ID NO. 66) data set numbering:

```
HCPCPAPELLGGPSVFIFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQPVFSWYVDGVEVHTAKMLTKPR
25                          50                          75

EEQFNSTYRVVSVLPIQHQDWLNGKEFKCKVNNKALPAPIEKTISKAKGQPREEPQVYVLPPPREELSKND
100                         125                         150

TVSLTCLVKGFYPPDIAVEWQSNGQPEPENKYKTTPPQLDSDGSYFLYSKLSVDKSRWQQGNTFTCSVMHE
175                         200                         225

ALHNHYTQKSLSKSPGK.
252
```

All existing and potential mutations follow this numbering system. Potentially beneficial mutations within bovine C_H2 based on residue frequency analysis include N85H, R109P, T116L and H126N. Potentially beneficial mutations within murine C_H2C_H3 based on residue frequency analysis include V48T, I64V, K66Q, S104V, S127N, F130L, F176Y, W186S, Q200P, V211L and A224Q. Potentially beneficial mutations within human C_H2C_H3 based on residue frequency analysis include K72Q, Y98F, L111Q, S126N and V202Q.

Example 9

N-terminal Sequencing of Plant Produced Antibody

N-terminal Sequencing was performed on the 36/41 IgG1 molecule for further characterization of the transgenic material. Both IgG1 from hybridoma material and transgenic plant material was sequenced. Light chain was not separated from heavy chain before sequencing. Purified 36/41 monoclonal IgG1 from hybridoma cell lines and from transgenic plant material was dialyzed against H_2O, frozen and lyophilized to powder. Approximately 50 pmoles of each protein was sent to the Utah State University Biotechnology and Genomics Research Center for N-terminal sequencing. Sequencing was performed on a 10 pmol scale using an Applied Biosystems (Foster City, CA) Procise Sequencer at a sampling rate of 4 Hz and detector scale of 1 AUFS. A total of 10 cycles were performed on each protein to confirm the first 10 amino acids.

The light chain gave a consistently stronger signal in successive sequencing cycles, allowing for the discrimination of heavy and light chain amino acids. The N-

terminal sequences of the IgG1 molecules were not provided prior to data analysis to insure unbiased interpretation of the data. Errors are likely the result of the limited amount of material and the presence of residual glycine from the purification protocol. Results are tabulated in Table 13. In the case of the transgenic IgG1 material, the endoplasmic reticulum signal sequence was processed precisely as anticipated.

Table 13 Results of N-terminal sequencing performed on the 36/41 IgG1 molecule produced by murine hybridoma cells and transgenic corn material.

	36/41 hybridoma VL	36/41 hybridoma VH	36/41 transgenic plant VL	36/41 transgenic plant VH
Actual Sequence	ENVLTQSPAI (SEQ ID NO. 73)	EVQLQQSGPE (SEQ ID NO. 75)	ENVLTQSPAI (SEQ ID NO. 77)	EVQLQQSGPE (SEQ ID NO. 79)
N-term. Sequencing	-VRLTQSPAI (SEQ ID NO. 74)	EVQLQQSGPE (SEQ ID NO. 76)	-LVLTQSPAI (SEQ ID NO. 78)	E-QLQQSGPE (SEQ ID NO. 80)

In light of the detailed description of the invention and the examples presented above, it can be appreciated that the several aspects of the invention are achieved.

It is to be understood that the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention, and that many alternatives, modifications, and variations will be apparent to those of ordinary skill in the art in light of the foregoing examples and detailed description. Accordingly, this invention embraces all such alternatives, modifications, and variations.